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(54) Title: FUNCTIONAL INTERACTIONS BETWEEN GLIAL S-100$_B$ AND CENTRAL NERVOUS SYSTEM SEROTONERGIC NEURONS

(57) Abstract

A method for stimulating the production of S-100$_B$ in a subject is provided by administering an effective amount of a 5-HT--$_{1A}$ receptor agonist, the agonists including receptor, including an antipeptide antibody to a functional domain of a 5-HT--$_{1A}$ receptor. Additional methods for stimulating the growth of central serotonergic neurons are provided, by contacting such neurons with an effective amount of S-100$_B$, or analog or derivative thereof, and the growth of central serotonergic neurons is inhibited by contacting the neurons with an effective amount of an inhibitor of S-100$_B$ production or action. Furthermore, diseases associated with decreased central serotonergic innervation or activity including autism, depression, anxiety, biological rhythm-based sleep disorder, and cortical brain damage may be treated by such methods. Diseases associated with increased central serotonergic innervation but ineffective astroglial S-100$_B$ release, such as Down's Syndrome and Alzheimer's Disease may be treated by up-regulating astroglial 5-HT--$_{1A}$ receptors, followed by stimulation of S-100$_B$ production or release using a 5-HT--$_{1A}$ receptor agonist, including an anti-peptide antibody to a functional domain of a 5-HT--$_{1A}$ receptor.

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FUNCTIONAL INTERACTIONS BETWEEN GLIAL S-100$_B$ AND CENTRAL NERVOUS SYSTEM SEROTONERGIC NEURONS

BACKGROUND OF THE INVENTION

Field of the Invention

The invention in the field of neuroscience and medicine relates to the discovery and use of a cortical growth factor S-100$_B$ as trophic for cortical and serotonergic neurons in the brain, as well as the use of 5-HT-$_1$A agonists, S-100$_B$ and derivatives thereof, to induce corticol or serotonergic growth, stimulation or regeneration, for therapeutic and/or diagnostic applications, *in vivo, in situ and/or in vitro*. Additionally, the present invention relates to the use of 5-HT-$_{1A}$ antagonists, also including peptides corresponding to functional domains of 5-HT-$_1$A receptors, and antibodies thereto, which up regulate central serontonergic neurons, which subsequent stimulation is enhanced or provided by such up regulation.

Description of the Background Art

Serotonergic neurons, which release serotonin (5-hydroxytryptamine, 5-HT) as a neurotransmitter, play a key role in the general maturation of the brain. Changes in the innervation density of serotonergic nerve fibers would be expected to induce changes in the maturation of "target" neurons with which the serotonergic fibers communicate.

Binding studies with appropriately labelled ligands initially revealed the existence of two major types of serotonin receptors in the brain, termed 5-HT$_1$ and 5-HT$_2$, and later pointed to the existence of further subtypes, such as 5-HT$_{1A}$ and 5-HT$_{1b}$ (Peroutka, et al., *Feder. Proc.* 42:212-217 (1983); Pazos et al., *Brain Res.* 205:346 (1985)). Other types of 5-HT receptors (e.g., 5-HT$_3$ and 5-HT$_4$) have been described more recently (see: Whitaker-Azmitia et al., Eds., *Ann. N.Y. Acad. Sci.* v600 (1990)).

Serotonin neurons have been shown to regulate their own development, i.e. to "autoregulate" (Whitaker-Azmitia et al., *Neurosci. Lett.* 67:307-312 (1986)), due in part to release of growth factors by stimulation of 5-HT$_{1A}$ receptors on astrocytes (Whitaker-Azmitia et al., *J. Neurochem.* 46:1186-91

(1986)). Glial cells, such as astrocytes, have 5-HT receptors (Whitaker et al., *ibid.*). The conditioned medium from glial cells which had been exposed to a selective agonist for the 5-HT_{1A} receptor subtype stimulated cultured serotonergic neurons (Whitaker-Azmitia et al., *Brain Res.* 497:80-85 (1989)). These results suggest the existence of a soluble hippocampal SGF of glial origin.

A number of proteins have been implicated as neuronal growth factors. Nerve growth factor (NGF) appears to act as a CNS cholinergic growth factor (Hefti, *J. Neurosci.* 6:2155-2162 (1986)). Epidermal growth factor (EGF) has trophic effects on neuron-like PC-12 cells (Leonard et al., *Mol. Cell. Biol.* 7:3156-3167 (1987); Isobe et al., *J. Neurochem.* 43:1494-1496 (1984)). Insulin has been shown to mediate growth of cultured fetal neurons (Heindenreich et al., *Endocrinology* 125:1451-1457 (1989)). The protein $S\text{-}100_B$, which is composed of two β-subunits, stimulates neurite extension in cultured chick cortical neurons, hence its designation as a "cortical" growth factor (Kligman et al., *Proc. Natl. Acad. Sci. U.S.A.* 82:7136-7139 (1985)). Calmodulin has been shown to have substantial structural homology and a similar Ca^{2+} binding profile to $S\text{-}100_B$ (Isobe et al., *Endocrinology* 125:1451-1457 (1989)).

A family of proteins named S-100 was first isolated nearly 25 years ago (Moore, *Biochem. Biophys. Res. Commun.* 19:739-742 (1965)). The member of this family designated $S\text{-}100_B$ was previously known to promote neurite extension in vitro, in chick embryo cultures (Kligman et al., *Proc. Natl. Acad. Sci. USA* 82:7136-39 (1985)). S-100 production was stimulated in cultures of the rat astroglioma line, C6, by dibutyryl cyclic AMP (Labourdette et al., *Biochem. Biophys. Res. Comm.* 96:1702-09 (1965)). Furthermore, S-100 may be releasable from brain tissue (Shashoua et al., *J. Neurochem.* 42:1536-41 (1984)).

The well-known factor NGF has been found to have no effect of 5-HT sprouting in damaged hippocampal neurons (Kiedrowsk et al., *Eur. J. Neurochem.* 43:1494-1496 (1984)), or in cultured dopaminergic neurons (Dreyfus et al., *Brain Res.* 194: 540-547 (1980)).

During fetal brain development, S-100$_B$, as detected by S-100$_B$ specific antibodies, shows an intense yet transient rise in the midline raphe region, where the serotonergic neurons are developing (Van Hartesveldt et al., *J. Comp. Neurol.* 253:175-184 (1986)).

Finally, the human gene for the β subunit of S-100 has been mapped to the distal half of the long arm of chromosome 21 (Allore et al., *Science* 239:1311-1313 (1988)); this gene was recently shown to contain the cAMP responsive element, CRE, on the promoter region (Allore et al., *J. Biol. Chem.* 265:15537-15543 (1990)). Furthermore, recent studies have shown an increase in S-100 immunoreactivity in postmortem Alzheimer's Disease and Down's Syndrome brains (Griffin et al., *Proc. Natl. Acad. Sci. USA* 86:7611-7615 (1989)). Therefore, greater understanding of the physiological actions of S-100 and knowledge of which factors regulate its release are believed to be important for the understanding and treatment of both disorders.

A cDNA for the 5-HT$_{1A}$ receptor from the rat has been cloned and shown to have a coding region consisting of 1266 nucleotides corresponding to the 422 amino acids of 5-HT$_{1A}$ (Albert et al, *J. Biol. Chem.* 265:528 1990). The 5-HT$_{1A}$ receptor from rat has seven transmembrane domains, a large third cytoplasmic loop and is 89% homologous with the human gene. The rat 5-HT$_{1A}$ receptor encoding mRNA tissue distribution showed high levels in spectrum, hippocampus, thalamus, amygdala olfactory bulb, mesencephalon, medulla and hypothalamus. Detectable levels were seen in the cortex and basal ganglia, but not in the pineal and the pituitary (Albert et al., 1990). These results are in general agreement with the ^{3}H-8-OH-DPAT binding studies in the rat which showed widespread distribution of receptor labelling except in extrapyramidal areas (substantia nigra, caudate nucleus and the globus pallidus), cerebellum and habenula were levels were undetectable. (Pazos et al. *Brain Res.* 346:205-230 (1985); Verge et al. *J. Neurosci* 6:3474-3482 (1986)).

Site-directed antibodies based on synthetic peptides

have been used to study many previously uncharacterized proteins (Sutcliff et al. *Nature* 287:801-805 (1980); Walter et al, *Proc. Natl. Acad. Sci.* 77:5197-5200 (1980); Yu et al. *Journal of Cell Biology* 114:1217-1232 (1991)). This approach has the advantages of being applicable as soon as the cDNA sequence is known and that the antipeptide antibody can be directed against a specific short region of the molecule. In general, the short sequence is bound to a larger carrier protein to increase its antigenicity (Harlow et al. *Antibodies: A Laboratory Manual* Cold Spring Harbor Press (1988)). This approach has been applied to the 5-HT_{1A} receptor against a region of the third cytoplasmic loop. (Raymond et al. *Molecular Pharmacology* 36:015-0211 (1989)) raised an antibody (JWR 21) against the sequence 242-267 using keyhole limpet hemocyanin (KLH) as the carrier and (El Mestikawy et al. *Neurosci. Lett.* 118:189-192 (1990)) raised an antipeptide antibodies against a very similar sequence 243-268 using bovine serum albumin as carrier. Anatomical immunoautoradiographic studies with this latter antibody at 1/1000 dilution showed a "striking similar" distribution to that seen with ^{3}H-8-OH-DPAT binding (El Mestikaway et al. *Neurosci. Lett.* 118:189-192, 1990). In an immunocytochemical study, labeling of 5-HT perikarya and dendrites membranes was seen in the midbrain raphe nuclei (Sotelo et al. *Eur. J. Neurosci.* 2:1144-1154, 1990).

Citation of documents herein is not intended as an admission that any of the documents cited herein is pertinent prior art, or an admission that the cited documents is considered material to the patentabilty of the claims of the present application. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

SUMMARY OF THE INVENTION

The present invention relates to the discovery that S-100_B is a protein released upon stimulation of their 5-HT_{1A} receptors, which stimulation has further been found to promote the growth of central serotonergic neurons and/or cortical

neurons.

The present invention also relates to methods for modulating $S100_B$ effects *in vitro, in vivo* and *in situ*, as well as to diagnostic and/or therapeutic methods involving serotonergic neuronal growth and/or maintenance, through such modulation of $S100_B$.

The present invention is also directed, in one aspect, to a method for stimulating the production of $S\text{-}100_B$ in a subject, comprising administering a $S100_B$ stimulating effective amount of an agonist acting on the $5\text{-}HT_{1A}$ receptor, by the use of at least one 5-HT--$_{1A}$ agonist compound or anti-idiotype antibody to 5-HT--$_{1A}$ receptors which has the *in vivo* effect of stimulating $5\text{-}HT_{1A}$ receptors. Non-limiting examples of 5-HT--$_{1A}$ agonists useful in the present invention may include 5-hydroxytryptamine, 5-methoxytryptamine, buspirone, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), ipsaspirone, gepirone, SM-3997, lysergic acid, ipsapirone, diethylamide, and 5-HT--$_{1A}$ agonistic antibodies.

The stimulation of 5-HT--$_{1A}$ receptors may be made subsequent to up regulation of such receptors, which prior up regulation increases the stimulatory effect on cortical and/or serotonergic neurons, preferably serotonergic neurons, which prior up regulation can be accomplished according to known method steps, as known to those skilled in the art, based on the teachings and guidance presented herein.

Stimulation of $S\text{-}100_B$ or 5-HT--$_{1A}$ receptor stimulation in a subject may have the therapeutic or diagnostic effect of causing serotonergic neuron growth and/or stimulation, which may be suitable for treatment and/or diagnosis of at least one diseases involving serotonergic and/cortical neuronal degeneration, trauma or dysfunction, autism, depression, anxiety, biological rhythm-sleep, disorder, critical brain damage, tryptophan anaboic pathologies, monoamine oxidase pathologies, Down's Syndrome and Alzheimers disease, which may be related to brain immaturity, premature birth, aging, sleep apnea, loss of serotonin production developmental disorders, alcoholism, carcinoid syndrome and/or cocaine addiction.

The present invention is further directed to a method for inducing the growth and/or stimulation of central serotonergic neurons or serotonin release in a subject, comprising administering to the subject an effective amount of S-100_B, a functional derivative or analog thereof, or an agonist acting at the 5-HT_{1A} receptor, such as those described above and including nti-idiotype antibodies to at least one functional domain of a 5-HT--$_{1A}$ receptor, from a mammal, preferably a human.

The present invention also includes a method for inducing the growth and/or stimulation of central serotonergic neurons or serotonin, *in vitro or in vivo* by contacting the neurons with $S100_B$, a 5-HT--$_{1A}$ agonistic antibody, or a functional derivative or analog thereof. For treatment of diseases associated with dysregulation of serotonergic neurons, in vivo contacting is preferred.

Alternatively, the present invention involves a method for inhibiting the growth of central serotonergic neurons, comprising contacting the neurons with an effective amount of an inhibitor of S-100_B production or action. The inhibitor may be an antibody specific for S-100_B or a 5-HT_{1A} receptor antagonist, such as the non-limiting examples of spiperone and spiroxatine, or an 5-HT--$_{1A}$ receptor peptide corresponding to a functional domain of a 5-HT--$_{1A}$ receptor, or antibodies thereto, such as anti-peptide antibodies.

The present invention also relates to a method for treating a disease and/or pathology associated with decreased central serotonergic innervation or activity including decreased serotonin levels in a mammalian, preferably human, subject, by administering a serotonergic stimulating effective amount of S-100_B, a functional derivative thereof, or a 5-HT_{1A} agonist including, but not limited to an antibody to a functional domain of a 5-HT--$_{1A}$ receptor. Diseases for which this method is useful include diseases involving serotonergic and/cortical neuronal degeneration, trauma or dysfunction, autism, depression, anxiety, biological rhythm-sleep, disorder, critical brain damage, tryptophan anaboic pathologies, monoamine oxidase pathologies, Down's Syndrome and Alzheimers, which may

be related to brain immaturity, premature birth, aging, sleep apnea, loss of serotonin production developmental disorders, alcoholism, carcinoid syndrome and/or cocaine addiction.

The present invention is also directed to a method for treating a disease associated with increased central serotonergic innervation or activity in a subject, comprising administering an effective amount of an inhibitor of S-100_B production or action. Such inhibitors include an antibody specific for S-100_B 5-HT_{1A} receptors, and/or a 5-HT_{1A} receptor antagonist.

In another embodiment, the invention is directed to a method for stimulating serotonergic neuronal growth and/or stimulation in a subject having Alzheimer's disease comprising the steps of:

(a) up-regulating the expression of 5-HT-$_{1A}$ receptors on astroglial cells in the brain of the subject; and then

(b) stimulating the induction of the release of S-100_B in the subject according to the methods described above,

thereby stimulating the cortical neuronal growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a histogram showing the effects of EGF (0.5μg/ml), NGF (10 μg/ml), S-100_B (10 μg/ml) and insulin (100 μg/ml) added daily in 5-fold serial dilutions (most concentrated dose at right) on serotonin uptake. Each bar is the mean + S.E.M. (n=4) of [^{3}H]5-HT uptake as a percent of control. Control uptake was 5038 + 606 cpm for S-100_B/insulin and 6159 + 318 cpm for NGF/EGF.

Figure 2 is a graph showing the effects of a single application of S-100_B and calmodulin on [^{3}H]5-HT uptake capacity after 3 days. Final concentration is shown on the abscissa. Each point represents the mean + S.E.M. (n=4).

Figure 3 is a histogram showing a morphometric analysis of the total neurite length for individual 5-HT-immunoreactive neurons after 30 h of stimulation. The bars

represent the mean + S.E.M. for 10 neurons in each well (number shown under bars).

Figure 4 is a graph showing effects of native bovine S-100 and of media from astroglial cells stimulated with the selective 5-HT_{1A} agonist ipsapirone (GCM-IPS) on the growth of serotonergic neurons in culture as determined by selective uptake of ^{3}H-serotonin. Hatched bars indicate the effects of S-100 and GCM-IPS in the presence of an antibody to S-100 (final dilution 1/10,000). Each bar represents the mean ± S.E.M. of 4 cultures, derived from different litters. S-100, GCM-IPS and the antibody were all added at time of plating and the growth assessed 3 days later.

Figure 5 is a pictorial representation of antipeptide 5-HT--$_{1A}$ receptor antibody binding to a hippocampus in midbrain section of a rat brain at 13500 times wherein the label is associated with microtubules (MTB) and also bound along the outer plasma membrane (PLMB) neurons.

Figure 6 shows a schematic diagram of the 5-HT--$_{1A}$ receptor.

Figure 7 shows a graphical representation of a purified synthetic peptide corresponding to a portion of a functional domain of a 5-HT--$_{1A}$ receptor.

Figure 8 shows a graphical representation of radio-labeled anti-goat anti-rabbit Ig fragments to antipeptide antibodies of the present invention wherein labeling radioactivity it various antibody dilutions.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the discovery that a protein designated S-100_B or functional derivatives and/or analogs thereof, is a serotonergic specific growth factor (SGF) that is also induced by serotonin. Since this protein has no effect on cholinergic and noradrenergic neurons, nor on cells in the peripheral nervous system, S-100_B, or derivatives or analogs thereof, are specific for modulation of central serotonergic nerves.

The present invention also relates to the discovery

that S-100$_B$ is a growth factor released in response to 5-HT$_{1A}$ receptor stimulation as well as by serotonin. Serotonin neurons have been shown to autoregulate their own development. The present inventors first discovered that this autoregulatory circuit involves the release of a growth factor or factors induced by stimulation of 5-HT$_{1A}$ receptors *in vivo, such as on* astrocytes. Therefore, the present invention is directed in one aspect to the use of 5-HT$_{1A}$ agonists or antagonists as therapeutics, acting via the regulation and/or modulation of S-100$_B$ production and/or release from astroglial cells and/or serotonergic neurons.

The present invention is also directed to methods involving the use of the S-100$_B$ protein, which is a dimer of two β chains, and is found exclusively in the brain, in contrast to α-α or α-β dimers, which are also found outside the brain. Also included within the scope of the present invention are functional derivatives of the S-100$_B$ protein.

An "analog" of S-100$_B$ refers to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

By "functional derivative" is meant a "fragment," "variant," or "chemical derivative" of S-100$_B$, which terms are defined below. A functional derivative retains at least a portion of the function of the S-100$_B$ which permits its utility in accordance with the present invention, namely serotonergic or cortical growth factor activity.

A "fragment" of the S-100$_B$ refers to any subset of the molecule, or of the β chain, such as a shorter peptide.

A "variant" of the S-100$_B$ refers to a molecule substantially similar to either the entire peptide or a fragment thereof. Variant peptides may be conveniently prepared by direct chemical synthesis of the variant peptide, or by recombinant DNA technology, using well known method steps. Amino acid sequence variants of the S-100$_B$ molecule can also be prepared by mutations in the DNA. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence. Any combination of

deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity, without placing the sequence out of reading frame and preferably not creating complementary regions that could produce secondary mRNA structure (see EP Patent Application Publication No. 75,444). At the genetic level, these variants ordinarily are prepared by site-directed mutagenesis of nucleotides in the DNA encoding the S-100_B molecule, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. The variants typically exhibit the same qualitative biological activity as the naturally occurring analog. Additionally, or alternatively, variants have greater than 80% homology with the corresponding $S100_B$ proteins or fragments, such as 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%, while maintaining at least some HT-5_{1A} receptor modulating activity.

A "chemical derivative" of S-100_B contains additional chemical moieties not normally a part of the protein or peptide. Covalent modifications of the protein are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

Also included in the scope of the invention are salts of the proteins and peptides of the invention. As used herein, the term "salts" refers to both salts of carboxyl groups and to acid addition salts of amino groups of the protein or peptide molecule. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases such as those formed for example, with amines, such as triethanolamine, arginine, or lysine, piperidine, procaine, and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid.

By the term "agonist" is intended any chemical or biological substance capable of binding to a particular receptor, such as the 5-HT_{1A} receptor or binding site thereof, according to the present invention, which binding stimulates a biological response associated with the receptor. The term is intended to include an endogenous molecule which exerts its physiological action by receptor binding and triggering of a signal to a cell, as well as an exogenous agent which mimics the action of such an endogenous agonist. Thus, if a receptor is linked to a second messenger system that signals a positive response, such as, for example, the increased production and/or secretion of a protein growth factor, such as $S100_B$, or analog or functional derivative thereof, an agonist will induce production and/or secretion of the growth factor. In contrast, if a receptor is linked to a second messenger system which signals a negative response, such as a termination of cell growth, an agonist for that receptor will inhibit cell growth.

Agonists for 5-HT_{1A} receptors include, but are not limited to, 5-hydroxytryptamine (serotonin), 5-methoxytryptamine, buspirone (U.S. Patent 3,717,634), 8-hydroxydipropylamineotetralin, ipsaspirone (EPO Publication 129,128A), gepirone (U.S. Patent 4,423,049), SM?3997 (U.S. Patent 4,507,303), MDL 72832 (U.S. Patent 4,612.312) ipsapirone, lysergic acid diethylamide and anti-idiotype antibodies to one or more functional domains of a 5-HT-$_{1A}$ receptor. In addition, newer polycyclic aryl- and heteroarylpipera- zinyl imides with 5-HT_{1A}-binding and activating properties, such as WY-47,846 (cpd. 34) and other disclosed in Abou-Gharbia et al., *J. Med. Chem.* 31:1382-1392 (1988), may be useful in the present invention, which references are hereby entirely incorporated by reference.

In addition, an antibody, preferably a monoclonal antibody (MAb) to the 5-HT_{1A} receptor which by virtue of its epitope specificity stimulates a response, rather than inhibiting the binding of an agonist, termed an "agonistic antibody," is also an agonist as intended herein. The present invention is intended to encompass additional 5-HT_{1A} receptor agonists, routinely obtainable according to the present

invention, based on the teaching and guidance presented herein without undue experimentation.

By the term "antagonist" is intended a substance which is itself devoid of intrinsic pharmacologic activity and stimulates no biological response when bound to a receptor, but has the capacity to bind to a receptor and thereby inhibit binding of, or action of, an agonist. Typically, antagonists act by competing for agonist binding to a receptor.

Antagonists for 5-HT_{1A} receptors are known in the art and include, but are not limited to, spiperone and spiroxatine. In addition, an antibody specific for the 5-HT_{1A} receptor which does not have agonist activity, but inhibits binding or action of an agonist, is also an antagonist, as intended herein. In addition, newer polycyclic aryl- and heteroaryl-piperazinyl imides with 5-HT_{1A}-binding properties which would inhibit binding of endogenous agonists (Abou-Gharbia, M. et al., supra) may be useful as antagonists in the present invention.

In addition, a 5-HT--$_{1A}$ peptide, as described herein, or a 5-HT--$_{1A}$ antagonistic antibody to such peptides, preferably a monoclonal antibody (MAb) to the 5-HT_{1A} receptor which, by virtue of its epitope specificity, inhibits the binding of a 5-HT--$_{1A}$ agonist, termed an "antagonistic antibody," is also an antagonist as intended herein. The present invention is intended to encompass additional 5-HT_{1A} receptor antagonists, routinely obtainable according to the present invention, based on the teaching and guidance presented herein without undue experimentation.

Surprisingly, peptides corresponding to portions of functional or other domains, such as transmembrane domains, of 5-HT--$_{1A}$ receptors have also unexpectedly been discovered to act as 5-HT--$_{1A}$ receptor antagonists, as do 5-HT--$_{1A}$ receptor antagonists, as described herein.

The principles of receptors, agonists, and antagonists are described, for example, in Gilman et al., Goodman and Gilman's "The Pharmacological Basis of Therapeutics", Eighth Edition, Macmillan Publishing Co., New York, 1990, Berkow et al, eds., *The Merck Manual*, 6th edition,

Merck and Co., Rahway, N.J., 1992; *Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics*, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD. (1987), Katzung, ed. *Basic and Clinical Pharmacology*, Fifth Edition, Appleton and Lange, Norwalk, Conn. (1992), which references, and references cited therein, are entirely incorporated herein by reference.

Antibodies of the present invention are those which are specific for, and interact with, S-100_B, functional derivatives or analogs thereof, or with 5-HT-$_{1A}$ receptors and modulate the action of the S-100_B-serotonergic neuron autoregulatory system, e.g., as 5-HT-$_{1A}$ receptor agonists or antagonists.

The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, and anti-idiotypic (anti-Id) antibodies or fragments, analogs or derivatives thereof. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen.

In the context of the present invention, a "5-HT-$_{1A}$ antibody" refers to an antibody, as described herein, which binds or associates with a 5-HT-$_{1A}$ receptor and has the *in vivo* biological activity of stimulating or inhibiting the 5-HT-$_{1A}$ receptor to have the effect of inducing or inhibiting, respectively, serotonergic and/or cortical neuron growth and/or stimulation, which effect may be mediated by the release of S-100_B, and wherein the modulation of central serontonergic neurons is preferred.

Such antagonistic 5-HT-$_{1A}$ antibodies of the present invention are preferably generated against a synthetic peptide corresponding to a functional domain of a 5-HT_{1A} receptor, according to the following criteria.

(1) Functional domain of the receptor: The receptor is homologous to the beta-adrenergic receptor family and many of the various segments of the 5-HT_{1A} receptor can be inferred from the extensive work with the β-adrenergic receptor. The agonist binding site consists of a least two aspartate (asp)

residues within the 2nd and 3rd transmembrane regions (Dohlman et al. Biochemistry 26:2657-2663 (1987). Asp residues (#82 and 116) exist in a similar site in the 5-HT_{1A} receptor (Figure 6). A histidine (His) in the 3rd transmembrane site (#126) would provide the needed positive charge for 5-HT binding. The third cytoplasmic loop is believed to be the site of interaction with the G-proteins in the cytoplasm for regulation of the second messenger systems (Kobilka et al. Nature 329:205-230 (1988)). Therefore, peptide sequences can be selected indicate the anatomical location of various segments of the full molecule and determine the cellular distribution of particular functional regions.

As a non-limiting example, domains were selected in the 2nd external loop (S1A-170) and in the 3rd cytoplasmic loop (S1A-258) (Fig. 6).

(2) Hydrophilicity regions. The antigenic sites on a peptide can be approximated based on the hydrophilicity score which assumes that the greater the local hydrophilicity, the more antigenic the sequence (Hopp, T.P. Proc. Natl. Acad. Sci. USA 8:3824-3828 (1981)). This measure assigns a numerical value to the various amino-acids; for example K, R, D and E have a value of +3.00, W has a value of -3.4, and G and P have a value of 0. the calculated window average at a residue is calculated across 6 residues. The hydrophilicity score for A1A170 is shown in Table I.

(3) Two dimensional protein structure. There are three states in which a sequence can exist in a secondary structure of a protein molecule. These are beta sheets, alpha helix and turns (Chou et al. Biochem. 13:222-245 (1974); Chou et al. Adv. Enzymology 47:45-147 (1978)). In the latter state, it is assumed that the amino acids are most exposed.

As a non-limiting example, the above selected peptides both have a significant number of predicted turns (Table I shows results for S1A170).

Table I.

The hydrophilicity scope according to Hopp and Wood (1981) and the secondary structure prediction of Chou and Fasman algorithm are shown below for the nonpeptide A1A170 (Intelligenetics program). Notice that a turn structure is predicted for the region showing a high positive hydrophilicity value.

Position	Structure	-2	-1	0	+1	+2
170	-		***********P			
171	-		***********P			
172	Beta		**********M			
173	Beta		**********L			
174	Beta		*********G			
175	Beta		********W			
176	Beta			R		
177	Turn			T******		
178	Turn			P******************		
179	Turn			E**********************		
180	Turn			D**********************		
181	Turn			R**********************		
182	Turn			S**********************		
183	Turn			D**********************		
184	Turn			P*****************		
185	Turn			D**********		
186	Turn			A*****		
187	Turn		*	C		

(4) Charge Balance of the Protein. The net charge of a peptide sequence should be near neutrality. If the molecule is too highly charged it will present problems during the purification procedure after the peptide is synthesized. If the net charge is highly basic or acidic a cation or anion exchange resin can be used. Bio-rad AG-50 resin has been successfully used for very basic peptides. However, strong deviations from neutrality is also a problem during the attachment to KLB which should proceed at neutral pH (see below).

As a non-limiting example, S1A-170 has 6 charged residues and a net -2 charge while S1A-258 has 5 charged residues and a net + 1 charge.

(5) Amino Acid length. The sequence for an ideal peptide for antibody formation should have 15-20 amino acids. A strand of 6 amino acids is the lower limit for a recognition site whole more than 20 presents some additional problems with

the synthesis and structural considerations (Harlow, E. et al. Antibodies: A Laboratory Manual Cold Spring Harbor Press (1988)).

As a non-limiting example, both S1A-170 and S1A-258 have 17 residues.

(6) Phosphorylation and glycosylation sites. A protein molecule has many possible phosphorylation and glycosylation sites. These sites should be avoided in choosing a sequence unless a particular confirmation is sought. Antibodies have been raised against phosphorylated sequences but these antibodies have altered affinity for the un-phosphorylated site (See Czernik et al. Method Enzymol 201:264-283 (1991). Furthermore, a phosphorylated segment of the molecule often confers allosteric changes in the protein structure which may reduce the affinity for the peptide segment artificially produced. It can be appreciated, that sites adjacent to modified sites may be less desirable for the same reasons. The glycosylation sites are located on the Asparagine (ASN, N) residues at positions 10, 11 and 24. Three potential protein kinase C phosphorylation sites are located at 147-152, 227-232 and 341-345 and one additional phosphorylation site 251-253 (El Mestikawy et al, Neurochem. Res. 16:1-10 (1991)).

As a non-limiting example, neither S1A-170 or S1A-258 have phosphorylation nor glycosylation sites.

(7) Position of Cysteine. Cysteine (Cys, C) residues are commonly involved in disulfide bridges. For this reason it is advisable to avoid a Cys residue in the middle of a peptide sequence. There are 15 Cysteine residues in the 5-HT_{1A} receptor, 6 in the transmembrane regions, 3 in the 3rd cytoplasmic loop, four in the three extracellular loop and two in the C-terminal cytoplasmic tail. The cysteines in extracellular loop 1 and 2 have been proposed to form a disulfide link in the β_2-adrenergic receptor (Dohlman et al. Biochemistry 26: (1987)). Similar cysteines exist in the 5-HT_{1A} receptor (Figure 6). In designing the sequence, it is advisable to have a terminal cysteine residue in order to bind to KLH protein (Harlow, et al. Antibodies: A Laboratory Manual Cold

Spring Harbor Laboratory Press (1988). This can be either at the C- or N-terminus of the peptide depending on how the peptide is predicted to be exposed in the molecule. For instance, if the desired sequence is at the N-terminal end of the protein, then the cysteine should be placed at the C-terminal end of the peptide. In this way, the N-terminal end will be exposed after its attachment to the carrier protein.

As a non-limiting example, both peptides have an N-terminal Cys ut S1A-258 also contains a Cys in the center of the peptide at position 266.

(8) Homology with known proteins. In selecting a region of the 5-HT_{1A} receptor protein comparisons with 5-HT_2, 5-HT_{1C}, α_2 and B_2-adrenergic, muscarinic M1, and the D_2 receptor were performed (Juluis et al. Proc. Natl. Acad. Sci. USA 87:928-932 (1990); Bunzow et al. Nature 336:783-787 (1988)). Once a segment has been selected it should be compared to all known protein sequences. The sequence data bank searching program (Intelligenetics, Inc., Mt. View, CA-(415)-962-7300) we used is based on the algorithm of Welbur and Lipman. Sequence Homology was searched against the Protein Identification Resource data bank (PIR) and the Protein Sequence Data Bank which contains the translated European Molecular Biology Library (EMBL). Such a procedure will identify those known structures that could react with the antibody raised. This especially relevant when the protein in question has been identified in the same species chosen for study, and conversely, significant homology to an invertebrate protein is not necessarily a problem. High homology for the selected sequence of the same protein in different species is advantageous.

The present invention is also related to the production, by chemical synthesis or recombinant DNA technology, of 5-HT--$_{1A}$ receptor peptides, preferably as small as possible while still retaining sufficiently high affinity or interaction with G-protein coupled receptors to modulate, such as to inhibit binding to such receptors by 5-HT--$_{1A}$ receptor ligands.

5-HT--$_{1A}$ receptor peptides of the present invention may include 5-10 to 50-150 amino acid fragments. consensus

sequences or substitution sequences of 5-HT--$_{1A}$ receptors, including, but not limited to serotonin receptors (5-HT), cytomegalovirus 5-HT--$_{1A}$ receptors, endothelial cell 5-HT--$_{1A}$ receptors, testis 5-HT--$_{1A}$ receptors, and thoracic aorta 5-HT--$_{1A}$ receptors, and homologs thereof having a homology of at least 80% with at least one of transmembrane domains 1-7, as described herein. See, e.g., Probst et al *DNA and Cell Biology* 11:1-20(1992), which is entirely incorporated herein by reference.

Accordingly, a "5-HT--$_{1A}$ receptor peptide" of the present invention includes polypeptides having a "5-HT--$_{1A}$ receptor amino acid sequence" which substantially corresponds to at least one 4 to 50 amino acid fragment and/or consensus sequence of a known 5-HT--$_{1A}$ receptor or group of 5-HT--$_{1A}$ receptors, wherein the 5-HT--$_{1A}$ receptor peptide has homology of at least 80%, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% homology, while maintaining 5-HT--$_{1A}$ receptor modulating activity, wherein a 5-HT--$_{1A}$ receptor peptide of the present invention is not naturally occurring or is naturally occurring but is in a purified or isolated form which does not occur in nature. Preferably, a 5-HT--$_{1A}$ receptor peptide of the present invention substantially corresponds to a transmembrane domain of a 5-HT--$_{1A}$ receptor or group of 5-HT--$_{1A}$ receptors as a consensus sequence.

Also preferred are 5-HT--$_{1A}$ receptor peptides wherein the 5-HT--$_{1A}$ receptor amino acid sequence is 4-10 to 50 amino acids in length, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 or 150 amino acids, or any range therein.

An amino acid or nucleic acid sequence of a 5-HT--$_{1A}$ receptor peptide of the present invention is said to "substantially correspond" to another amino acid or nucleic acid sequence, respectively, if the sequence of amino acids or nucleic acid in both molecules provides polypeptides having biological activity that is substantially similar, qualitatively

or quantitatively, to the corresponding fragment of at least one 5-HT--$_{1A}$ receptor transmembrane domain, or which may be synergistic when two or more transmembrane domains, consensus sequences or homologs thereof are present.

Additionally or alternatively, such "substantially corresponding" sequences of 5-HT--$_{1A}$ receptor peptides include conservative amino acid or nucleotide substitutions, or degenerate nucleotide codon substitutions wherein individual amino acid or nucleotide substitutions are well known in the art.

Alternatively or additionally, substantially corresponding refers to 5-HT--$_{1A}$ receptor peptides having amino acid sequences having at least 80% homology or identity to an amino acid sequence of a human 5-HT--$_{1A}$ receptor, such as 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% homology or identity.

Accordingly, 5-HT--$_{1A}$ receptor peptides of the present invention, or nucleic acid encoding therefor, include a finite set of substantially corresponding sequences as substitution peptides or polynucleotides which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz, G.E. et al., *Principles of Protein Structure*, Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. For a presentation of nucleotide sequence substitutions, such as codon preferences, see Ausubel et al, *supra*, at §§ A.1.1-A.1.24, and Sambrook et al, *supra*, at Appendices C and D.

Conservative substitutions of a 5-HT--$_{1A}$ receptor peptide of the present invention includes a variant wherein at least one amino acid residue in the polypeptide has been conservatively replaced by a different amino acid. Such substitutions preferably are made in accordance with the following list as presented in Table IV, which substitutions may

be determined by routine experimentation to provide modified structural and functional properties of a synthesized polypeptide molecule, while maintaining the receptor binding, inhibiting or mimicking biological activity, as determined by known 5-HT--$_{1A}$ receptor receptor activity assays.

Table IV

Original Residue	Exemplary Substitution
Ala	Gly;Ser
Arg	Lys
Asn	Gln;His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala;Pro
His	Asn;Gln
Ile	Leu;Val
Leu	Ile;Val
Lys	Arg;Gln;Glu
Met	Leu;Tyr;Ile
Phe	Met;Leu;Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp;Phe
Val	Ile;Leu

Alternatively, another group of substitutions of 5-HT--$_{1A}$ receptor peptides of the present invention are those in which at least one amino acid residue in the protein molecule has been removed and a different residue inserted in its place according to the following Table V. The types of substitutions which may be made in the protein or peptide molecule of the present invention may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz et al., supra and Figs. 3-9 of Creighton, supra. Based on such an analysis, alternative conservative substitutions are defined herein as exchanges within one of the following five groups:

TABLE V

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: His, Arg, Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys); and
5. Large aromatic residues: Phe, Tyr, Trp.

Conservative amino acid substitutions according to the present invention, e.g., as presented above, are known in the art and would be expected to maintain biological and structural properties of the polypeptide after amino acid substitution. Most deletions and insertions, and substitutions according to the present invention are those which do not produce radical changes in the characteristics of the protein or peptide molecule. "Characteristics" is defined in a non-inclusive manner to define both changes in secondary structure, e.g. α-helix or β-sheet, as well as changes in physiological activity, e.g. in receptor binding assays.

However, when the exact effect of the substitution, deletion, or insertion is to be confirmed one skilled in the art will appreciate that the effect of the substitution or substitutions will be evaluated by routine screening assays, either immunoassays or bioassays to confirm biological activity, such as receptor binding or modulation of ligand binding to the corresponding 5-HT--$_{1A}$ receptor. See, e.g., Maranges et al., eds., for example, a substituted polypeptide typically is made by site-specific mutagenesis of the peptide molecule-encoding nucleic acid, expression of the mutant nucleic acid in recombinant cell culture, and, optionally, purification from the cell culture, for example, by immunoaffinity chromatography using a specific antibody on a chemically derivatized column or immobilized membranes or hollow fibers (to absorb the mutant by binding to at least one epitope).

A preferred use of this invention is the production, by chemical or recombinant DNA technology, of 5-HT--$_{1A}$ receptor peptides, preferably as small as possible while still retaining sufficiently high affinity for binding to, or association with, 5-

HT--$_{1A}$ receptors. By production of 5-HT--$_{1A}$ receptor peptides including smaller fragments or variants of such transmembrane domains, one skilled in the art, using known binding and inhibition assays, can readily identify the 5-HT--$_{1A}$ receptor peptides capable of binding minimizing or modulating G-protein coupled receptors using known methods.

Accordingly, 5-HT--$_{1A}$ receptor peptides may include consensus sequences and/or fragments of at least one of transmembrane domain 1-7 of one or more 5-HT--$_{1A}$ receptors, which 5-HT--$_{1A}$ receptor peptides do not occur naturally, and/or which are provided in an isolated and/or purified form not found in nature.

Consensus peptides of 5-HT--$_{1A}$ receptor peptides of the present invention may include peptides which are distinct from known 5-HT--$_{1A}$ receptor sequences in critical structural features, but which are derived from consensus sequences of homologous 5-HT--$_{1A}$ receptor transmembrane domains 1-7. Such consensus peptides may be derived by molecular modeling, optionally combined with hydrophobicity analysis and/or fitting to model helices, as non-limiting examples. Such modeling can be accomplished according to known method steps using known modeling algorithms, such as, but not limited to, ECEPP, INSIGHT, DISCOVER, CHEM-DRAW, AMBER, FRODO and CHEM-X. Such algorithms compare transmembrane domains between related G-protein coupled receptors, determine probable energy-miminized structures and define alternative consensus polypeptide fragments.

Such consensus peptides or fragments of 5-HT--$_{1A}$ receptors may then be synthesized or produced recombinantly, in order to provide 5-HT--$_{1A}$ receptor peptides according to the present invention which mimic, modulate or inhibit binding of ligands to G-protein coupled receptors. 5-HT--$_{1A}$ receptor ligands, in the context of the present invention, refer to biological molecules that bind 5-HT--$_{1A}$ receptors *in vitro*, *in situ* or *in vivo*, and may include hormones, neurotransmitters, viruses or receptor binding domains, thereof, opsins, rhodopsins, nucleosides, nucleotides, coagulation cascade factors, odorants or pheremones, toxins, colony stimulating factors, platelet activating factors, neuroactive peptides, neurohumors, or any biologically active

compounds, such as drugs or synthetic or naturally occurring compounds.

In the context of the present invention, 5-HT--$_{1A}$ receptor peptides of greater than 15-25 amino acids are preferred such that the 5-HT--$_{1A}$ receptor peptides are able to span the lipid bilayer.

Additionally, modified amino acids or chemical derivatives of amino acids of consensus or fragments of 5-HT--$_{1A}$ receptors proteins, according to the present invention may be provided, which polypeptides contain additional chemical moieties or modified amino acids not normally a part of the protein. Covalent modifications of the peptide are thus included within the scope of the present invention. Such modifications may be introduced into a 5-HT--$_{1A}$ receptor peptide by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

Preferably, once 5-HT--$_{1A}$ receptor functional domain peptides meeting the above criteria are determined, then such peptides can be synthesized according to known method steps, wherein solid phase synthesis is preferred (see. e.g., Harlow and Lane, *supra;* Barany et al. The Peptides Analysis Synthesis Biology 2:(E. Gross and J. Meiehofer, Eds.) Academic Press, NY pp. 1-284 (1979)) or p-methyl-benzylhydrylamine polystyrene resin using hydroxybenzotriazole-activated esters of N-α-Boc protected amino acids on an Applied Biosystems, Inc. Model 430A automated peptide synthesizer

Monoclonal antibodies are a substantially homogeneous population of antibodies to specific antigens. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, Nature 256:495-497 (1975) and U.S. Patent No. 4,376,110. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and/or any subclass thereof. The hybridoma producing the mAbs of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo production makes this the presently preferred method of production. Briefly, cells from the individual hybridomas are

injected intraperitoneally into pristane-primed BALB/c mice to produce ascites fluid containing high concentrations of the desired mAbs. MAbs of isotype IgM or IgG may be purified from such ascites fluids, or from culture supernatants, using column chromatography methods well known to those of skill in the art. See Harlow *supra*; Ausubel, *supra*.

Chimeric antibodies are molecules having different portions derived from different animal species, or different Ig subclasses of the same or different species, such as those having variable region derived from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies and method steps for their production are known in the art (Cabilly et al, Proc. Natl. Acad. Sci. USA 81:3273-3277 (1984); Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984); Boulianne et al., Nature 312:643-646 (1984); Neuberger et al., Nature 314:268-270 (1985); Taniguchi et al., European Patent Application 171496 Robinson et al., International Patent Publication #PCT/US86/02269; Liu et al., Proc. Natl. Acad. Sci. USA 84:3439-3443 (1987); Better et al., Science 240:1041- 1043 (1988)). These references are hereby entirely incorporated by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An anti-Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the mAb with the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). For example, anti-Id antibodies to 5-HT---$_{1A}$ receptors are expected to provide 5-HT--$_{1A}$ agonists as described herein, which would this be included as 5-HT--$_{1A}$ agonistic antibodies.

An anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is

possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against S-100$_B$, for example, may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for a S-100$_B$ epitope.

The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to the epitope being evaluated, such as an S-100$_B$ epitope, and possessing biological activity of S-100$_B$.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and F(ab')$_2$, which are capable of binding antigen. Fab and F(ab')$_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)).

It will be appreciated that Fab and F(ab')$_2$ and other fragments of the antibodies useful in the present invention may be used according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')$_2$ fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody which can also be recognized by that antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one, or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The preferred animal subject of the present invention is a mammal. By the term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

By the term "treating" is intended the administering to subjects of S-100$_B$, a functional derivative thereof, serotonin, or an agonist or antagonist of the 5-HT$_{1A}$ receptor, for purposes which may include prevention, amelioration, or cure of the diseases discussed below.

For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Preferred routes for administration of substances which do not cross the blood-brain barrier (such as proteins and larger peptides) to subjects with fully developed blood-brain barriers include intracranial and intracerebroventricular (i.c.v.) routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

According to the present invention, 5-HT-$_{1A}$ receptor agaonists (for serontonergic or cortical neuron stimulation through induction of S-100$_B$ release) or 5-HT-$_{1A}$ receptor antagonists or anti-S100$_B$ antibodies (for up regulation of serontonergic and/or cortical neurons), a described herein, or as would be clear to one skilled in the art, based on the teaching and guidance presented herein, may be administered prenatally, neonatally or to an adult. For prenatal administration, the antibody is given systemically to the pregnant female or is introduced *in utero*, for example, into

the amniotic cavity. For neonatal treatment, an antibody is administered systemically, for example, by intravenous or intraperitoneal injection. The antibody can cross the blood-brain barrier and enter the brain from the circulation in a young individual in whom the blood-brain barrier is not completely formed, as is well-known to those of skill in the art. In an individual with a fully formed blood-brain barrier, as in an adult, in order to be effective, the antibody, according to the present invention, must be administered intracranially (i.c.), preferably into the cerebral ventricles (i.c.v.) via a cannula, using methods well-known in the art. See, e.g., Berker, *supra*, Goodman, *supra*, Avery, *supra* and Katzung, *supra*, which are entirely incorporated herein by reference, including all references cited therein.

In addition to the pharmacologically active compounds, such as the 5-HT_{1A} receptor agonists and antagonists, S-100_B and functional derivative thereof, or antibodies, the present invention contemplates pharmaceutical preparations which may also or alternatively contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those preparations which can be administered orally and which can be used for the preferred type of administration, such as tablets, dragees, and capsules, and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection or orally, contain from about 0.01 to 99 percent, preferably from about 20 to 75 percent of active compound(s), together with the excipient. See, e.g., Berker, *supra*, Goodman, *supra*, Avery, *supra* and Katzung, *supra*, which are entirely incorporated herein by reference, including all references cited therein.

Suitable excipients are, in particular, fillers such as saccharides, for example, lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example, tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin,

tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl-starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol.

A number of major developmental disorders involve central serotonergic systems. Autism and Down's Syndrome (DS) are associated with altered serotonergic forebrain innervation, as seen upon postmortem examination of brains from patients with this disorder (Anderson, G.M. et al., Ann. N.Y. Acad. Sci. 600:331-342 (1990)). Hyperserotoninemia has been observed in autistic children and an antibody specific for the human cortical 5-HT_{1A} receptor has been identified in an autistic patient (Anderson, G.M. et al., supra). The present invention provides a means for treating an individual affected by autism or DS in early developmental stages, for example, while in utero. Thus, for example, S-100_B, a functional derivative thereof, or 5-HT_{1A} agonist is administered to a pregnant woman carrying a fetus diagnosed as having autism or DS.

Alzheimer's Disease (AD) shares a number of pathophysiological similarities to DS and is also associated with Chromosome 21 (Allore et al., 1988, supra). Brains of AD patients have increased levels of S-100_B, in particular in astrocytes (Griffin, W.S.T. et al., supra), which is not normally releasable. AD patients also have increased serotonergic innervation. As a result of the increased presence of 5-HT--, astroglial 5-HT--$_{1A}$ receptors are down-regulated.

The present invention provides a method for treating AD by manipulating the serotonergic system to cause release of S-100_B to stimulate growth of cortical and/or serotonergic neurons.

The method, and all other therapeutic methods of the present invention, including, but to limited to stimulation of S-100_B, 5-HT--$_{1A}$ receptor stimulation in a subject may have the therapeutic or diagnostic effect of causing serotonergic neuron

growth and/or stimulation, which may be suitable for treatment and/or diagnosis of diseases involving serotonergic and/cortical neuronal degeneration, trauma or dysfunction, autism, depression, anxiety, biological rhythm-sleep, disorder, critical brain damage, tryptophan anaboic pathologies, monoamine oxidase pathologies, Down's Syndrome and Alzheimers disease, which may be related to brain immaturity, premature birth, aging, sleep apnea, loss of serotonin production developmental disorders, alcoholism, carcinoid syndrome and/or cocaine addiction, is directed to optionally first up-regulating the astroglial 5-HT--$_{1A}$ receptors in order to render them sensitive to 5-HT--$_{1A}$ or exogenous agonists, and then to treating with the agonists in order to stimulate S-100$_B$ release.

Up-regulation of the 5-HT--$_{1A}$ receptors is accomplished by any of a number of means known in the art for depleting central 5-HT--$_{1A}$ or blocking its action. Such means include depleting stored serotonin from nerve terminals by agents such as reserpine, fenfluramine or methylene deoxymethamenpetamine (MDMA) (Whitaker-Azmitia, P.C. et al., Eds, Ann. N.Y. Acad. Sci., Vol. 600 (1990)), dietary changes which lower central serotonin levels, such as a tryptophan-deficient diet, or drugs which inhibit 5-HT--$_{1A}$ biosynthesis such as parachlorophenylalanine. Alternatively, since central 5-HT--$_{1A}$ release is dependent on intact brain corticosteroids, any treatment which blocks brain corticosteroid levels (for example, synthesis inhibitors such as metapyrone or aminoglutethamide) or corticosteroid action (such as corticosteroid antagonists which are well-known in the art) would also be useful in up-regulating 5-HT--$_{1A}$ receptors. Treatment with 5-HT--$_{1A}$ receptor antagonists, including 5-HT--$_{1A}$ receptor peptides, or antipeptide antibodies, or anti-Id mAbs such as those described herein, may also achieve the same or similar effects. Such treatment must be performed for a period of time ranging from about 3 days to about 4 weeks prior to stimulation of central serotonergic neurons, as described herein. Depending on the drug or means chosen to up-regulate the receptors, and the age, weight and health of the subject, one of ordinary skill in the art will be able to determine the appropriate dose and time course of

treatment.

Once the 5-HT--$_{1A}$ receptors have been appropriately up-regulated, resulting in regained sensitivity to agonist action, the treatment method of the present invention involves providing to the brain a 5-HT--$_{1A}$ receptor agonist as described herein, preferably by the oral or parenteral route, in a dose range of about 1 μg/kg to about 10 mg/kg, for a duration of about 3 days to about 4 weeks, in order to release the S-100$_B$ accumulating in the astroglia. The released S-100$_B$ then acts as a cortical growth factor to stimulate growth of cortical neurons deficient in AD.

In fact, any pathological process associated with loss of cortical neurons and/or serotonergic neurons or their activity, or lack of normal maintenance of serotonergic innervation, can be treated according to the methods of the present invention. For example, such neuronal loss may accompany normal aging. The present invention is thus directed to a method of treating neuronal loss in an aging individual with S-100$_B$, a functional derivative thereof, or a 5-HT$_{1A}$ agonist which stimulates S-100$_B$ production by astroglial cells in situ. If necessary, as described for AD, prior treatment may be used to up-regulated 5-HT--$_{1A}$ receptors in order to allow stimulation of S-100$_B$ release.

Affective disorders, in particular depression, are diseases with an important serotonergic component. In fact, the mode of action of many effective antidepressant drugs is considered to occur via inhibition of 5HT$_{1A}$ re-uptake, thus prolonging the availability of 5-HT--$_{1A}$ to act on post-synaptic 5-HT--$_{1A}$ receptors. Whereas uptake blockade can occur within minutes of treatment, therapeutic benefits are typically seen only after prolonged (e.g. 3 weeks) of antidepressant therapy (see, for example, Gilman et al., supra). This difference in time courses suggested to the present inventors that depression involves alterations in levels of serotonergic innervation and synapse formation. Thus, according to the present invention, stimulation of serotonergic neuronal growth by treatment with S-100$_B$, a functional derivative thereof, or a 5-HT$_{1A}$ agonist which stimulates endogenous S-100$_B$ production, can be used to treat depression. A subject in need of treatment is administered an effective amount of S-100$_B$, a functional

derivative thereof, or a 5-HT_{1A} agonist. In adults, the treatment with S-100_B, or functional derivatives which do not cross the blood-brain barrier is by i.c.v. infusion. In neonates or immature subjects in whom the blood-brain barrier is not fully formed, the protein or derivative may be administered systemically. 5-HT_{1A} agonists, most of which readily cross the blood-brain barrier, are administered systemically.

Antidepressant agents have also been useful in the treatment of anxiety and obsessive-compulsive disorders (Gilman et al., supra), indicating the involvement of the serotonergic system in these states. In addition, 5-HT-$_{1A}$ is involved in synchronization of biological rhythms, and dysregulation may result in sleep disorders (Wauquier, A. et al., Ann. N.Y. Acad. Sci. 600:447-459 (1990)). According to the present invention, serotonin, S-100_B, a functional derivative thereof, or a 5-HT_{1A} agonist may be used to treat anxiety and sleep disorders due to its action as an inducer of central serotonergic neuronal growth, as described above for treatment of depression.

Schizophrenia, a major psychiatric illness, is increasingly looked upon as a developmental disorder of the dopaminergic (DA) system wherein central DA activity is increased (Seene, P., Pharmacol. Rev. (1982)). The DA system appears to interact physiologically with serotonergic neurons in a way that stimulation of 5-HT-$_{1A}$ results in decrease in DA levels. Therefore, stimulation of growth of serotonergic neurons by administration of S-100_B or a functional derivative thereof, or stimulation of S-100_B release, according to the present invention may be useful in preventing or reversing the effects of enhanced dopaminergic activity, and thus the development of schizophrenia. In a preferred embodiment, S-100_B, serotonin or precursor thereof or a 5-HT_{1A} agonist which stimulates astroglial production and/or secretion of S-100_B is administered to a pregnant female carrying a fetus at risk for schizophrenia, either systemically or by intrauterine introduction.

Due to its action as a central cortical and/or serotonergic growth factor, S-100_B promotes the growth and maintenance of cortical neurons, most of which utilize glutamate

as their neurotransmitter. According to the present invention, S-100_B, a functional derivative thereof, or a 5-HT_{1A} receptor agonist may be used to induce repair of cortical neurons following cortical brain damage, such as that associated with traumatic head injury or stroke. Treatment of a subject in need of repair of cortical neurons is performed as described above for other diseases.

According to the methods of the present invention, the amounts and regimens for the administration of S-100_B and functional derivatives thereof, 5-HT_{1A} agonists and antagonists, and anti-S-100_B agonistic and antagonistic antibodies can be determined readily by those with ordinary skill in the clinical art of treating the particular disease. While individual needs vary, determination of optimal ranges of effective amounts of each component is witnin the skill of the art. Typical dosages comprise about 0.001 to 100 mg/kg body wt such as 0.01-50, 0.05-20, 0.1 to 10 or 1 to 10 mg/kg. The preferred dosages comprise 0.1 to 10 mg/kg body wt.

As an alternative form of treatment to the administration of S-100_B or 5-HT_{1A} agonists, the present invention also contemplates the implantation or transplantation of an astroglial cell capable of producing S-100_B to a subject having a deficit in such cells or having a genetic lesion rendering such cells non-functional, for example, non-responsive to 5-HT_{1A} receptor stimulation. Such implanted cells may be derived from fetal or adult brain or may be a long term cell line, such as the C6 cell line (Labourdette, G. et al., supra), which is maintained in culture. Such cells can be implanted in specific target regions in the brain in order to stimulate serotonergic cell growth or cortical cell growth, as discussed above. (See, also: Azmitia, E.C. et al., Eds., Ann. N.Y. Acad. Sci.., vol.495 (1987)).

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

S-100$_B$ IS A CNS SEROTONERGIC GROWTH FACTOR

METHODS

Brains from rat embryos (14 days; Taconia Farms, Germantown, NY) were dissected and the mesencephalic raphe region was removed and dissociated in Ca^{2+}/Mg^{2+} free Eagles' minimal essential medium (MEM; Sigma) by repeated trituration. The cells in complete medium (MEM supplemented with non-essential amino acids and 5% fetal calf serum) were plated in 96-well plates (Nunc Labware) coated with poly-L-lysine (25 μg/ml, Sigma) at initial plating densities (IPD) of 1 x 10^6 cells/cm^2.

After 3 days of incubation the cultures were washed and neuronal growth was assessed by the specific fluoxetine-sensitive high-affinity uptake of 50nM ^{3}H-serotonin for 20 min at 37°C in fresh MEM with 10^{-5}M pargyline (Azmitia, E.C. et al., Neuroscience 20:47-63 (1987)).

For morphometric analysis, cells were plated on 8-well glass culture chamber slides (Miles Scientific) for 30h, fixed with ice-cold 4% paraformaldehyde and reacted with a specific 5-HT antibody (1/4000 dilution; Incstar, Stillwater, MI) and an avidin-biotin secondary (Vector Labs). The 5-HT immunoreactive neurons were analyzed using a Bioquant computer imaging system. The slides were coded and all measures taken blind by a naive observer. On each well, 10 areas (0.5 mm^2) were randomly selected and the largest 5-HT-IR neuron in the field measured for somal area and neurite length (Azmitia, E.C., et al., 1987, supra).

The following protein preparations were dissolved in MEM, sterilized with 0.2 μm Uniflo filter units and added to the cultures at a 1/10 dilution: Human S-100$_B$, calmodulin (East-Acres Biologicals, Southbridge), NGF prepared from mouse submaxillary glands (Mobley, W.C. et al., Biochem. 15:5543 (1979)), EGF and insulin (Boehringer-Mannheim Biochemicals).

RESULTS

The daily chronic addition of S-100$_B$ produced a dose-dependent increase in 5-HT uptake capacity with maximal effect (171% of control) at 3.2 ng/ml and a significant increase (150% of control) seen at the lowest concentration tested of 1 pg/ml (Figure

1). Analysis of variance of the data of Figure 1 yielded the following values:

Comparison	df	F	p
S-100$_B$/Insulin	23	11.353	< 0.001
NGF/EGF	22	6.148	< 0.05

A post-hoc Tukey test showed + = $p < 0.05$ and ++ = $p < 0.01$.

Neither insulin, NGF nor EGF produced an increase in uptake capacity. A single application of S-100$_B$ at initial cell plating produced, after 3 days of incubation, a dose-related stimulation in uptake capacity (maximal stimulation of 85% at 5 ng/ml) while calmodulin was without effect (Figure 2). Analysis of variance for the data in Figure 2 showed df=23, F=24.3, and $p < 0.0001$. Students t-test control comparisons showed + = $p < 0.05$ and ++ = $P < 0.01$.

The uptake of [^{3}H]5-HT by the cultured serotonergic neurons is used as an index of total surface area of the neuron, However, it is possible that S-100$_B$ might interact directly with the %-HT protein transporter and produce a change in the uptake of [^{3}H]5-HT independent of a increase in neurite length. Two experiments tested this possibility:

(1) S-100$_B$ (16 ng/ml) added for 15 min. to 3-day-old mesencephalic cultures immediately before [^{3}H]5-HT uptake produced no change (3140 + 337 vs 3719 + 177, S-100$_B$ and control, respectively). This indicates that S-100$_B$ is not interacting directly with the 5- HT transporter.

(2) S-100$_B$ was added at initial plating for 30h to mesencephalic cultures and the 5-HT neurons were immunocytochemically stained. The neurite length of 5-HT-IR neurites was increased by 35% and 47% at two doses (16 and 3.2 ng/ml, respectively) of S-100$_B$ (Figure 3; ANOVA showed F = 22.69; df = 20 and $p < 0.0001$. Post-hoc Tukey test showed ++ = $p < 0.01$). No change in somal area was found. This increase in neurite length after 30h is consistent with the increase observed in [^{3}H]5-HT uptake by the cultured mesencephalic neurons after 3 days of incubation.

DISCUSSION

The results indicate that S-100$_B$ is an SGF. In contrast, EGF, insulin and calmodulin were not found to produce any stimulation, indicating that the SGF activity of S-100$_B$ was not a byproduct of its Ca^{2+} binding potential nor was it due to a general mitogenic potential.

S-100 in the brain is an astroglial specific protein (Isobe, T. et al., supra). Recent results have shown that the SGF properties of 5-HT$_{1A}$-stimulated, glial cell conditioned medium is blocked by treatment with an anti-S-100 antibody (see Example II, below).

It also possible that the soluble SGF detected several weeks after a 5,7-DHT lesion in adult hippocampus (Azmitia, E.C., et al., Soc. Neurosci. Abstr. 12 (1986)) and believed to be responsible for collateral sprouting of serotonergic nerves is also S-100$_B$.

EXAMPLE II

STIMULATION OF ASTROGLIAL 5-HT$_{1A}$ RECEPTORS RELEASES THE SEROTONERGIC GROWTH FACTOR, S-100$_B$

To test whether S-100 was the factor released by 5-HT$_{1A}$ receptor stimulation, astroglial cells were stimulated in primary culture with 100 nM ipsaperone (IPS), a 5-HT$_{1A}$ receptor agonist, and collected the conditioned media (GCM-IPS). We then added the GCM-IPS to primary cultures of serotonin neurons, with and without the addition of an antibody to S-100, and assessed the effects on neuronal growth.

METHODS

Primary astroglial cultures were derived from newborn (1 to 3 day old) Sprague-Dawley rat pups as previously described (Whitaker-Azmitia, P.M. et al., Brain Res. 497:80-85 (1989)). After one week in culture, cultures were rinsed twice with Weymouth's medium containing 5 μg/ml insulin and 0.5 mg/ml albumin. The cells were left to incubate at 37°C. for twelve hours before replacing media with fresh serum-free media containing 100 nM ipsapirone, a selective 5-HT$_{1A}$ receptor agonist. After 24 hr, the media (referred to as GCM-IPS) was collected and stored at -70°C until tested in neuronal cultures.

The growth-promoting properties of native bovine S-100 (10-1000 ng/ml and of GCM-IPS (diluted 1 to 500) were compared. The S-100 was obtained from East Acres Biologicals, Southbridge, MA (guaranteed >99% homogenous by SDS-PAGE).

S-100 or GCM-IPS, or each of these in the presence of a polyclonal antibody to S-100 (Accurate Chemical, Westbury, NY; final dilution 1/10,000) were added at the time of neuronal plating. The polyclonal antibody had been characterized in our laboratory and shown to be positive for immunocytochemical staining of astrocytes in culture and brain).

Mesencephalic neuronal cultures were prepared from Sprague-Dawley rat embryos at 13-14 days of gestation (obtained from Hilltop Breeding Laboratories) as previously described Azmitia, E.C. et al., Neuroscience 20:47-63 (1987). After three days in culture, neuronal growth was assessed by measurement of specific serotonin re-uptake capacity. This indicator has been shown to be a reliable measure of in vivo innervation density and of the maturational state of specific neurons in culture (Azmitia, E.C., 1987, supra; Currie, D.N. et al., Brain Res. 199: 473-81 (1980); Dreyfus, C.F. et al., Brain Res. 128:124-139 (1977)). Briefly, cultures were incubated for 20 minutes with MEM containing 1% glucose and ^{3}H-serotonin (26 Ci/mmole, New England Nuclear; final concentration 50 nM) with or without 50 nM fluoxetine. After removing the radiolabel, the cultures were allowed to dry and 200 microliters of absolute ethanol was added for one hour. Then, 150 μl of the sample was placed into 7 ml of Liquiscint for counting in a Beckman Liquid Scintillation Counter (40% efficiency). Test cultures were pre-incubated with S-100 or GCM-IPS for one hour before uptake was measured.

To visualize astroglial cultures after exposure to serum-free media with or without 100 nM ipsapirone, cultures were rinsed twice with Tris-buffered saline (TBS) at 4°C before incubation with a polyclonal antibody to a specific astroglial marker, glial fibrillary acidic protein (GFAP) (Accurate Chemicals; final dilution 1/800 in TBS with 0.2% Triton and 0.1% normal swine serum) for 2 hrs at 37°C. After rinsing with TBS, the cultures were stained using the avidin/biotin method prepared as Vectastain

(Vector Labs) with final visualization using diaminobenzidine.

RESULTS

Both S-100 (500 ng/ml) and GCM-IPS (diluted 1/500) produced an increase in the ^{3}H-serotonin uptake capacity of the cultures after 3 days of exposure, but not when applied acutely. This stimulation was blocked by incubation, at the time of plating, with a polyclonal antibody to S-100 at a 1/10,000 dilution (Figure 8; df=5; f=1497, $p < .0001$; for individual values, $p < .001$). Application of either the antibody alone or 0.5 nM ipsaperone(the maximum final concentration in the neuronal cultures after addition of GCM-IPS) was without significant effect. The majority of the activity is thought to reside in the β subunit of S-100 since S-100_B (β-β dimer) is more active than S-100_A (β-α dimer).

The morphological alterations were characterized by an increase in process-bearing cells and an increased colonization of the cells. These changes were consistently observed in all eight primary cultures (ie. from eight different litters of animals) used to derive GCM.

DISCUSSION

It had been previously demonstrated that serotonergic neurons regulate their own growth through activation of a 5-HT_1 receptor (Whitaker-Azmitia, P.M. et al., Neurosci. Lett. 67:307-312 (1986)). The present inventors found that astroglial cells contain high levels of 5-HT_1 receptors in the immature state (Whitaker-Azmitia, P.M. et al., J. Neurochem. 46:1186-91 (1986)) and that activation of a subtype of these receptors, the 5-HT_{1A} receptor, leads to secretion into the medium of a factor which can stimulate serotonergic maturation in dissociated tissue culture preparation (Whitaker-Azmitia, P.M. et al., Brain Res. 497:80-85 (1989)).

Based on the above results, 5-HT_{1A} receptors on brain astroglial cells appear to be involved in the release of S-100. Therefore, S-100 provides at least one means by which serotonin can autoregulate development of serotonergic nerves.

During fetal brain development, S-100, as detected by antibodies, shows an intense yet transient rise in the midline raphe region, where the serotonin cells are developing (Van Hartesveldt, C.J. et al., J. Comp. Neurol. 253:175-184 (1986)).

Since in the process of producing and/or releasing S-100, the astroglial cells attain a mature morphology, the present results suggest a functional interaction between astrocytes and neurons during development, whereby both cell types mature through the action of the astroglial 5-HT_{1A} receptor.

The observed morphological change has also been seen after activation of other receptors linked to generation of cyclic AMP, such as the β-adrenergic receptor, the stimulation of which is linked to the release of nerve growth factor (NGF) (Schwartz, J.P. et al., Naunyn Schmeideberg's Arch. Pharmacol. 300:123-129 (1977)). The parallels of these two systems is noteworthy: both involve receptor stimulation coupled to cAMP production which results in astroglial morphological changes and finally release of a growth factor

In summary, of the several growth factors tested, only chronic S-100_B showed enhancement (maximal at 3.2 ng/ml is 171%) after 3 days of incubation of the [^{3}H]5-HT uptake capacity by serotonergic neurons. A single application at initial plating of S-100_B (maximal at 5 ng/ml is 185%), but not calmodulin, increased the development of the [^{3}H]5-HT uptake capacity by the cultured serotonergic neurons. Morphometric analysis of cultured 5-HT immunoreactive (IR) neurons showed an increase (135 and 147%) in neurite length 30 h after S-100_B application of 16 and 3.2 ng/ml (respectively). The results indicate that S-100_B functions as a serotonergic growth factor in the mammalian brain.

Stimulation of astroglial 5-HT_{1A} receptor causes astroglial cells to acquire a more mature morphology and to release a factor (or factors) which promotes growth of serotonergic neurons. By using an antibody- blocking approach, it has been conclusively demonstrated that one of the growth- factors released is the astroglial-specific protein S-100. This may be a particularly important observation, in view of studies implicating S-100 in both Down's Syndrome and Alzheimer's Disease, as discussed above.

EXAMPLE III

ANTIPEPTIDE ANTIBODIES AGAINST THE 5-HT_{1A} RECEPTOR

According to the present invention, a method is provided for selecting two new sites for anti-5-HT_{1A} receptor antibody recognition against the 5-HT_{1A} receptor: S1A-170 (aa 170-186) and S1A-258 (aa 258-274). These antibodies recognized a protein brand of approximate molecular weight of 49,000. they showed excellent staining in rat neonatal and adult brain and in adult monkey brain at dilutions as low as 1/10,000. Similarities and differences with the distribution with ^{3}H-8-OH-DPAT binding were seen. Most importantly, clear laminar labelling was seen in those areas known to have high 5-HT_{1A} binding such as the hippocampus and cortex. In addition, selective cells were labeled in areas thought to have little or no 5-HT_{1A} receptors, such as the cerebellum and striatum. Light cellular labeling was apparent in many areas. In the hippocampus, polymorphic interneurons were stained. There was less intense labeling in the granule cell layer. The neurons in the rostral dorsal raphe nucleus were clearly stained in the monkey. In the cerebellum, labelling was very high in the neonate but mainly confined to glial cells in the adult. High label was seen in epithelial cells lining the brain and the ventricular system. Tanocytes were labelled in the third ventricle near the median eminence. Staining was seen to astroglial cells in many brain areas and astroglial cultures. Ultrastructural studies revealed heavy staining in occasional primary dendrite shafts in the hippocampus midbrain. The label was associated with the Microtubules (MTB) and in patches along the outer plasma membrane (PLMB) of neurons (Fig. 5 shows dendrite 1t 13,500 X).

The selection of the peptide domain of the 5-HT_{1A} receptor against which the antibodies were raised was made the basis of several criteria, as follows:

(1) Functional domain of the receptor: The receptor is homologous to the beta-adrenergic receptor family and many of the various segments of the 5-HT_{1A} receptor can be inferred from the extensive work with the β-adrenergic receptor. The agonist binding site consists of a least two aspartate (asp) residues

within the 2nd and 3rd transmembrane regions (Dohlman et al. Biochemistry 26:2657-2663 (1987). Asp residues (#82 and 116) exist in a similar site in the 5-HT_{1A} receptor (Figure 6). A histidine (His) in the 3rd transmembrane site (#126) would provide the needed positive charge for 5-HT binding. The third cytoplasmic loop is believed to be the site of interaction with the G-proteins in the cytoplasm for regulation of the second messenger systems (Kobilka et al. Nature 329:205-230 (1988)). Therefore, peptide sequences can be selected indicate the anatomical location of various segments of the full molecule and determine the cellular distribution of particular functional regions. We selected domains in the 2nd external loop (S1A-170) and in the 3rd cytoplasmic loop (S1A-258) (Fig. 6).

(2) Hydrophilicity regions. The antigenic sites on a peptide can be approximated based on the hydrophilicity score which assumes that the greater the local hydrophilicity, the more antigenic the sequence (Hopp, T.P. Proc. Natl. Acad. Sci. USA 8:3824-3828 (1981)). This measure assigns a numerical value to the various amino-acids; for example K, R, D and E have a value of +3.00, W has a value of -3.4, and G and P have a value of 0. the calculated window average at a residue is calculated across 6 residues. The hydrophilicity score for A1A170 is shown in Table One.

(3) Two dimensional protein structure. There are three states in which a sequence can exist in a secondary structure of a protein molecule. These are beta sheets, alpha helix and turns (Chou et al. Biochem. 13:222-245 (1974); Chou et al. Adv. Enzymology 47:45-147 (1978)). In the latter state, it is assumed that the amino acids are most exposed. The selected peptides both have a significant number of predicted turns (Table I shows results for S1A170).

(4) Charge Balance of the Protein. The net charge of a peptide sequence should be near neutrality. If the molecule is too highly charged it will present problems during the purification procedure after the peptide is synthesized. If the net charge is highly basic or acidic a cation or anion exchange resin can be used. Bio-rad AG-50 resin has been successfully used for very

basic peptides. However, strong deviations from neutrality is also a problem during the attachment to KLB which should proceed at neutral pH (see below). S1A-170 has 6 charged residues and a net -2 charge while S1A-258 has 5 charged residues and a net + 1 charge.

(5) Amino Acid length. The sequence for an ideal peptide for antibody formation should have 15-20 amino acids. A strand of 6 amino acids is the lower limit for a recognition site whole more than 20 presents some additional problems with the synthesis and structural considerations (Harlow, E. et al. Antibodies: A Laboratory Manual Cold Spring Harbor Press (1988)). Both S1A-170 and S1A-258 have 17 residues.

(6) Phosphorylation and glycosylation sites. A protein molecule has many possible phosphorylation and glycosylation sites. These sites should be avoided in choosing a sequence unless a particular confirmation is sought. Antibodies have been raised against phosphorylated sequences but these antibodies have altered affinity for the un-phosphorylated site (See Czernik et al. Method Enzymol 201:264-283 (1991). Furthermore, a phosphorylated segment of the molecule often confers allosteric changes in the protein structure which may reduce the affinity for the peptide segment artificially produced. It can be appreciated, that sites adjacent to modified sites may be less desirable for the same reasons. The glycosylation sites are located on the Asparagine (ASN, N) residues at positions 10, 11 and 24. Three potential protein kinase C phosphorylation sites are located at 147-152, 227-232 and 341-345 and one additional phosphorylation site 251-253 (El Mestikawy et al, Neurochem. Res. 16:1-10 (1991)). Neither S1A-170 or S1A-258 have phosphorylation nor glycosylation sites.

(7) Position of Cysteine. Cysteine (Cys, C) residues are commonly involved in disulfide bridges. For this reason it is advisable to avoid a Cys residue in the middle of a peptide sequence. There are 15 Cysteine residues in the 5-HT_{1A} receptor, 6 in the transmembrane regions, 3 in the 3rd cytoplasmic loop, four in the three extracellular loop and two in the C-terminal cytoplasmic tail. The cysteines in extracellular loop 1 and 2 have been proposed to form a disulfide link in the β_2-adrenergic

receptor (Dohlman et al. Biochemistry 26: (1987)). Similar cysteines exist in the 5-HT_{1A} receptor (Figure 6). In designing the sequence, it is advisable to have a terminal cysteine residue in order to bind to KLH protein (Harlow, et al. Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press (1988). This can be either at the C- or N-terminus of the peptide depending on how the peptide is predicted to be exposed in the molecule. For instance, if the desired sequence is at the N-terminal end of the protein, then the cysteine should be placed at the C-terminal end of the peptide. In this way, the N-terminal end will be exposed after its attachment to the carrier protein. Both peptides have an N-terminal Cys ut S1A-258 also contains a Cys in the center of the peptide at position 266.

(8) Homology with known proteins. In selecting a region of the 5-HT_{1A} receptor protein comparisons with 5-HT_2, 5-HT_{1C}, α_2 and B_2-adrenergic, muscarinic M1, and the D_2 receptor were performed (Juluis et al. Proc. Natl. Acad. Sci. USA 87:928-932 (1990); Bunzow et al. Nature 336:783-787 (1988)). Once a segment has been selected it should be compared to all known protein sequences. The sequence data bank searching program (Intelligenetics Inc., Mt. View, CA-(415)-962-7300) we used is based on the algorithm of Welbur and Lipman. Sequence Homology was searched against the Protein Identification Resource data bank (PIR) and the Protein Sequence Data Bank which contains the translated European Molecular Biology Library (EMBL). This research will identify those known structures that could react with the antibody raised. This especially relevant when the protein in question has been identified in the same species chosen for study, and conversely, significant homology to an invertebrate protein is not necessarily a problem. High homology for the selected sequence of the same protein in different species is advantageous. Neither selected sequence has any significant homology with any other published receptor or mammalian protein. Interestingly, S1A-170 has a 94% with the human 5-HT_{1A} receptor, while S1A-258 has only 44% homology with the human 5-HT_{1A}. In the case S1A170, the methionine (-1,3 hydrophilicity) at position 172 is replaced by an isoleucine (-1.8 hydrophilicity) which should have little

consequence to the antigenicity of the sequence.

Synthetic Peptides

The peptides were made according to method steps as described in detailed previously (Yu et al., 1991). Briefly, the two sequences selected, CSH 228 (S1A-170-86:H-Pro-Pro-Met-Leu-Gly-Trp-Arg-Thr-Pro-Glu-Asp-Arg-Ser-Asp-Pro-Asp-Ala-Cys-(SEQ ID No:1); and CSH229(S1A-258-274:H-Pro-Gly-Ser-Gly-Asp-Trp-Arg-Arg-Cys-Ala-Glu-Asn-Arg-Ala-Val-Gly-Cys-(SEQ ID No:2), were synthesized by the solid phase methods (Barany et al. The Peptides Analysis Synthesis Biology 2:(E. Gross and J. Meiehofer, Eds.) Academic Press, NY pp. 1-284 (1979)) or p-methyl-benzylhydrylamine polystyrene resin using hydroxybenzotriazole-activated esters of N-α-Boc protected amino acids on an Applied Biosystems, Inc. Model 430A automated peptide synthesizer. A modified small-scale (0.1 mmole) rapid-cycle was used. Couplings were done in dimethylformamide and dichloromethane as solvents, and unreacted peptide was capped with acetic anhydride. The side chain-protected amino acids were: Arg (Mts); His (BOM); Thr (Bzl); Cys (4-CH3-Bzl); Trp (CHO); Ser (Bzl; Glu (OBzl); and Asp (cHex). Double coupling was necessary for several amino acids such as Trp, Leu, Thr, Glu, Ser, Cys and Val.

The peptides were deprotected and cleaved from the resin with liquid FH at -10°C for 2 h in the presence of 5% (v/v) anisole and 5% (v/v) dimethyl sulfide. The peptide was precipitated with ethyl either, and solubilized in 6M Guanidine HCl @ 10 mg/ml (41 ml). The formyl group was removed from the indole portion of Trp by treatment with HF. The sample was cooled to 0°C in a salt ice bath in a round bottom flask with stirring. Ethanolamine was added at a final concentration of 1M (2.5 ml) and stirred for 4 hr. at 0°C. The temperature was critical with the pH > 8 since a low temperature prevents the cyclization of glutamic acid and aspartic acid. The reaction was quenched by reducing the pH < 7.0 with [HCL] (Baker, HPLC grade). The sample was filtered (0.45μm pores, Nylon 66, Schleicher & Schuell) prior to HPLC purification.

The solution was subjected to HPLC using a Waters Delta Prep 3000 instrument on a column (4.9 x 30 cm) of 300 Å, C_{18} silica (Waters) and eluted with 0.1% (w/v) trifluoroacetic acid with a

linear gradient to 60% acetonitrile (Burdick and Jackson). Both peptides (CSH228 and 229) eluted at approximately 23 min. at approximately 49% acetonitrile. The Mass Spectrometry data showed a [M+H]+ ion with no other adducts or deletion products (Figure 7). Final yields were 50 mg of 228 and 26 mg of 229.

Antibody Preparation

The peptides, CSH 228 and 229 were coupled to keyhole limpet hemocyanin (Sigma) via maleimidobenzoyl-N-hyroxysuccinimide (Pierce Chemical Co., Rockford, IL) as described (Harlow et al. Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press (1988). Briefly, the Keyhole limpet hemocyanin (KLH) was dissolved in PBS (10 mg/ml) M-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Pierce, N22312) was dissolved in dimethylformamide (Burdick and Jackson, Baxter, N.J.). The MBS solution was very slowly added to the KLH solution, one drop at a time while stirring and the mixture allowed to mix for 30 min at RT. The unbound MBS was removed by filtration on Sephadex G25 in PBS (Pharmacia). The peptides were dissolved in PBS at 10 mg/ml and added slowly to the MBS/KLH solution. The pH was adjusted to 7.2 and the mixture is allowed to stir for 3 hr at RT. The solution was extensively dialyzed against PBS in the cold room over night. The protein concentration in the dialysate was determined with the Bio Rad protein microassay and the solution adjusted to 1 mg/ml.

Antisera against the complexes were raised as follows. The complexes (0.5 mg) in 0.5 ml of phosphate-buffered saline (PBS) was mixed with 0.5 ml of Freund's complete adjuvant and 300 μl injected subcutaneously in four spots on the back of adult female New Nezland white rabbits weighing 8 1/2 and 8 lbs. that were certified specific pathogen free (Hare Maryland, Hewitt, NJ). Booster injections were give with incomplete adjuvant at 2 week internals with 200μl incomplete adjuvant at 2 spots on the back. Additional booster injections were given at 2 week intervals until reaching maximum serum titer. The first bleed of 6 ml was made from the central artery of the ear 3 days after the second boost. Once the maximum titer was reached (fourth boost), full bleed of 15-20 ml were made. The blood was kept at 5°C and spun once at low speed for 10 min and the serum spun a second time in Eppendorf

tubes for 10 min before being aliquoted and frozen.

Serum antibody titer was determined by radioimmunoassay. Wells of 96-well polyvinylchloride microtiter plates (Falcon Microtest III) were coated with 50 μl of the appropriate peptide (1 mg/10 ml) for at least 3 hr at RT. The plates were washed 3 times with PBS and unbound sites were saturated with 200 μl of 3% (w/v) bovine serum albumin (BSA). Dilutions of immune and preimmune serum were added to wells at concentration from 1/50 to 1/50,000 for at least 1h at RT. The plates were washed 3 times with PBS and 50,000 cpm of ^{125}I-labelled goat anti-rabbit IgG F(ab)'^{2} (NEX 167, NEN) added per well and allowed to include at RT for 1 hr. After three washes, the specific activity of each serum sample was determined by counting the radioactivity for 1 min in a gamma radiation counter (Beckman, Gamma 5500 B). The results for S1A1709 and S1A258 are shown in Fig. 8.

Gel Electrophoresis and Immunoblotting

Electrophoresis was performed on 1mm 12.5% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulfate using the buffer system of Laemmli (1977). 20 and 40 ug of tissue from hippocampus was run in each well along with 5ul of standard (Bio Rad biotinylated SDS-PAGE standards, low range cat # 161-0306). The hippocampus was removed from a young Long Evans female rat (100-150 gm; Charles River, Kingston, NY) and immediately frozen in liquid nitrogen. A single hippocampus was transferred to lysis buffer (see below) containing 2% SDS. The tissue was immediately homogenized by hand in a 1.5 ml polypropylene eppendorf tube (pellet pestle with disposable tube, #74920, Kontes, N.J.) and allowed to sit for 15 min on ice before spinning in a microfuge Eppendorf Microcentrifuge 5414) at 5°C for 15 min. The supernatant was collected and assayed for protein amount using a commercial dye-binding microassay (Bio Rad). Routinely, 3ul were added to 1ml of solution and read on a spectrophotometer at wave length 595 nm. The solution was adjusted to a final concentration of 2 mg/ml. The supernatant was mixed with an equal amount of sample buffer (Laemmli), β-mercaptoethanol added to a final concentration of 10% (V/v) and the sample boiled for 5 min. The gel was run for 5 hr.

at 100 volts on a vertical gel electrophoresis apparatus. Proteins were transferred electrophoretically to nitrocellulose using a Bio-Rad Trans Blot Cell overnight at 50 volts in the cold room (Towbin et al., 1979 & 1984).

The nitrocellulose was incubated with antiserum at dilutions from 1/100 to 1/10,000 in 0.1% (v/v) Tween-20 (Sigma) in Tris buffered (0.1M, pH 7.4) saline (0.9%) solution (TTBS). It is our experience that the best results were obtained with our antibodies at dilution of 1/1000 to 1/5000. The antibody at lower dilutions (1/250) gave increase background and less sensitivity. The avidin-biotin peroxidase procedure was used to identify the protein band as described by Vector Laboratories. Briefly, the nitrocellulose sheets were cut to include a standard and the appropriate rows and washed in small 75mm disposable Petri dishes. The nitrocellulose strips were incubated with the antisera for at least 2 hr at 40°C (the strips would be left with antisera for several days at RT or in the Cold room). The strips were rinsed three times in TTBS for a total of 10 min on a shaker between biotinylated secondary (30 min incubation), the ABC reagents (20 min incubation), and the DAB peroxide reaction. The strips were first incubated with freshly filtered diaminobenzidine (5 mg/20 ml TBS; Sigma) and 0.2% Nickel ammonium sulfate for 5 min at RT and the H_2O_2 added at a final concentration of 0.01% (v/v). Boehringer-Mannheim produces biotinylated reagents that are easier and cheaper to use than Vector Stains products but we have not yet compared their sensitivity. One major band (49.5 kdal) and two minor bands (42.0 and 37.0 kdal) were stained with S1A170 at a dilution of 1/1000.

Lysis Buffer (Draeta et al. Cell 54:17-26 (1988)) was made from highest grade chemicals from Sigma (St. Louis) and Boehringer-Mannheim: 50mM Tris pH 7.4; 150 mM NaCl; 1% NP-40; 10mM EDTA; 1mM $MgCl_2$; 1 mM $CaCl_2$; 10% Glycerol; 400 μM Sodium Orthovanadate; 50 mM NaFluoride; 50mg/l PMSG (phenylmethane sulfonylfluoride) from 10mg/ml isopropanol; 1 mg/leupeptin; 10 mg/l Soybean Trypsin Inhibitor; 1mg/l Aprotinin and 10 mg/l of TPCK (L-1-chiloro-3-[4-tosylamido]-4]phenyl-2-butanone) from 3 mg/ml of ethanol.

Immunocytochemistry at the light and ultrastructural level. Neonatal (1-2 weeks) and adult female rats were perfused with a variety of fixatives and prepared for immunocytochemistry according to our published procedures (Azmitia et al. J. Neuroscience 3:2083-2090 (1983). Rats (Sprague-Dawley, Female, Taconic Breeders, 220 gm) and monkeys (Macaca Fascicularis, female, Charles River Breeding Laboratory, 3.3 kg) were perfused, through the ascending aorta with 4% praformaldehyde, 2% glutaraldehyde, 4% formaldehyde plus 0.1% glutaraldehyde or 3.25% acrolein with 2% praformladehyde at 20°C and 0.1% $MgSO_4$ in 0.1M phosphate buffer (pH 7.4) at 20°C. The glutaraldehyde and acrolein fixatives were continued after 10 min with the same solution with only paraformaldehyde (total perfusion volume was 100 ml for neonate, 250 ml for the adult rats and 1500 ml in the monkey) for an additional 20 min. The brains were profixed at 5°C for at least 4 hr before being processed for immunocytochemistry. Thirty-micrometer sections of the hippocampus and brainstem were cut on a Vibratome (Oxford).

The primary anti-serum and the secondary sera were diluted in 0.1 M Tris buffered (pH 7.4) saline (0.85%) containing 1% normal sheep serum and 0.1% Triton X-100. The sections were incubated for 18-72 hr at 5°C followed by 2 hr at room temperature (RT) in antipeptide antibody serum at a dilution of 1/1000-1/10,000. The sections were then processed with the elite Vector stain ABC-kit as directed by the manufacturer. The reaction was run for 2 min at RT in 0.05% 3,3-diaminobenzidine containing 0.2% nickel ammonium sulfate in 0.1 M Tris-buffered (pH 7.4) saline (Azmitia et al. J. Neuroscience 3:2083-2090 (1983)) followed for 5 to 10 min at RT in the same solution with the addition of 0.01% hydrogen peroxide. The sections for electron microscopy were viewed after postfixing for 1 hr at 20°C in 2% osmium tetroxide containing 1.5% potassium ferricyanide in 0.1 M phosphate buffer (pH 7.2) and then block stained in 0.5% uranyacetate at 5°C for 30 min. Ultrathin sections were taken for the surface of Epon/Araldite-embedded tissue slices and views on the electron microscope without further heavy metal staining.

Advantages

Specificity of the antibody raised against synthetic peptides is increased compared to using the full molecule since a specific region of the structure is targeted. Regions with high homologies with other molecules can be avoided. A functional region (3rd cytoplasmic loop) or a structural portion (second extracellular loop) can be selected for study (Fig. 6). The antibody peptide can be produced as soon as the primary cDNA structure has been demonstrated.

High titers with the antipeptide antibody can be obtained because the peptide is bound to a carrier protein that provides highly immunogenic sites for T-cell receptor binding. We were able to obtain RIA binding of the native peptide over 100 fold that seen in the preimmune serum at dilution of 1/10,000 (see Fig. 8).

Anatomical and cellular localization of the receptor can be performed in neonatal and adult rats as well as adult monkey. All fixation solutions produced good results. The best staining of CNS was in neonates and this confirms the ligand binding studies which have shown higher values for the 5-HT_{1A} receptor during early prenatal periods (Bar-Peled et al. Neurosci. Lett. 127:173-176 (1991); Daval et al. Intern J. Neurosci 5:171-180 (1987); Whitaker-Azmitia et al. Develop. Brain Res. 33:285-295 (1987)). The most interesting observation was the specific cellular staining observed in neurons, astrocytes, tanocytes, tanocytes and epithelial cells. The complex distribution was not suggested by earlier autoradiographic studies using ligand binding. The great advantage of a receptor antibody lies in its adaption of ultrastructural immunocytochemical localization (see Fig. 5).

Isolation of the receptor by immunoaffinity purification has been reported previously with an antipeptide antibody to the 5-HTLA receptor (Raymond et al. Molecular Pharmacology 36:015-021 (1989)). The provides a rapid and sensitive method to isolate a single protein fraction. The receptor can also be concentrated by the method of immunoprecipitation (EL Mestikawy et al. Neurochem. Lett. 118:189-192 (1990); see also Harlow et al. Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press (1988)).

Western analysis of the amount of the 5-HT_{1A} receptor can be performed with both the S1A170 and S1A258 antibodies that we have raised.

Characterization of the antipeptide antibody must establish that the native protein is selectively recognized, such that the following considerations are preferably addressed.

This requires demonstrating a specific cellular staining pattern in immunocytochemical studies. However, as discussed by El Mestikawa et al. Neurochem Res. 16:1-10 (1991), the distribution of receptor staining with antibodies may not be completely consistent with the radioautographic distribution seen with ligand binding. This is explained by the observation that ^{3}H-8-OH-DPAT, for instance does not recognize the receptor if it is not linked to a G-protein. The antibody, on the other hand, can recognize all the receptor molecules even those in transit from the cell body to the dendritic plasma membrane via microtubules (see Fig. 5).

Immunostaining of a single band in Western analysis is usually considered an indication of the general specificity of an antibody. However, this requires careful manipulation of the antibody dilution based and preparation of the tissue sample. Protein fragments or aggregation of the molecule can result in several bands on a Western even if the antibody only recognizes a single protein. For this reason, we use a special lysis buffer and treat the tissue with reducing and denaturing conditions (such as B-mercaptoethanol and boiling).

Immunoprecipitation and immunoaffinity purification of a single protein that functions as an active receptor with the appropriate characteristics is the best criteria that the native protein is labeled by the antibody. In studies with transfected COS-7 cells, the antibody JWR21 (242-267) was shown to precipitate the [^{125}I]N_3-NAPS photoaffinity labelled receptor (Raymond et al. Molecular Pharmacology 36:015-021 (1989)). The preimmune serum, the antigenic peptide block JWR21 antibody or an antibody from a non-overlapping region (268-293) were all unable to precipitate the receptor. In the study by El Mestikawy et al. Neurosci. Lett. 118:189-192 (1990), the 5-HT_{1A} receptor antibody against 243-268

precipitated the binding sites of ^{3}1H-8-OH-DPAT when protein A-sepharose CL-4B was added. No influence of the antiserum alone was seen in the binding.

The synthesis was performed with a 430A ABI sequencer. The alternative is TFMSA cleavage which can be done at the bench.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific

embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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WHAT IS CLAIMED IS:

1. A method for stimulating the production or release of S-100$_B$ in an animal, comprising administering to said subject an effective amount of an agonist acting on a central 5-HT$_{1A}$ receptor.

2. A method according to claim 1, wherein said agonist is selected from the group consisting of 5-hydroxytryptamine, 5-methoxytryptamine, buspirone, 8-hydroxydipropylaminotetralin, ipsaspirone, gepirone, SM23997, lysergic acid diethylamide, and an agonistic antibody.

3. A method for stimulating growth of central serotonergic neurons in an animal, comprising administering to said subject a serotonergic neuron stimulating effective amount of S-100$_B$, a functional derivative thereof, or an agonist acting at the 5-HT$_{1A}$ receptor.

4. A method according to claim 3 or 5, wherein said agonist is selected from the group consisting of 5-hydroxytryptamine, 5-methoxytryptamine, buspirone, 8-hydroxydipropylaminotetralin, ipsaspirone, gepirone, SM23997, lysergic acid diethylamide, and 5-HT--$_{1A}$ agonistic antibody.

5. A method for stimulating growth of central serotonergic neurons comprising contacting said neurons with an effective amount of S-100$_B$ or a functional derivative thereof.

6. A method according to any of claims 1-5, wherein said contacting is *in vitro*.

7. A method according to any of claims 1-5 wherein said contacting is *in vivo*.

8. A method for inhibiting the growth of central serotonergic neurons comprising contacting said neurons with an effective amount of an inhibitor of S-100$_B$ production or action.

9. A method according to claim 8, wherein said inhibitor is an antibody specific for S-100$_B$ or a 5-HT--$_{1A}$ receptor antagonistic antibody or peptide.

10. A method according to claim 9, wherein said antibody is a monoclonal antibody.

11. A method according to claim 9, wherein said antibody is a polyclonal antibody.

12. A method according to claim 8, wherein said inhibitor is a 5-HT_{1A} receptor antagonist.

13. A method according to claim 12, wherein said 5-HT_{1A} antagonist is selected from the group consisting of spiperone and spiroxatine.

14. A method for treating a disease associated with decreased central serotonergic innervation or activity in a subject, comprising administering to said subject an effective amount of S-100_B, a functional derivative thereof or a 5-HT_{1A} agonist.

15. A method according to claim 14, wherein said agonist is selected from the group consisting of 5-hydroxytryptamine, 5-methoxytryptamine, buspirone, 8-hydroxydipropylamineotetralin, ipsaspirone, gepirone, SM23997, lysergic acid diethylamide and a 5-HT-$_{1A}$ receptor agonistic antibody.

16. A method according to claim 14 wherein said disease is selected from a group consisting of autism, depression, anxiety, biological rhythm-based sleep disorder, and cortical brain damage.

17. A method for treating a disease associated with increased central serotonergic innervation or activity in a subject, comprising administering to said subject an effective amount of an inhibitor of S-100_B production or action.

18. A method according to claim 17 wherein said inhibitor is an antibody specific for S-100_B.

19. A method according to claim 17 wherein said inhibitor is a 5-HT_{1A} receptor antagonist.

20. A method for stimulating cortical or serotonergic neuronal growth or stimulation in a subject having Alzheimer's disease, comprising

(a) up-regulating the expression of 5-HT-$_{1A}$ receptors on astroglial cells in the brain of said subject; and then

(b) stimulating the induction of the release of S-100_B in said subject according to a method according to claim 1,

thereby stimulating said serotonergic neuronal growth.

21. A method according to claim 1, wherein said antagonist is a monoclonal 5-HT--$_{1A}$ receptor antibody or a 5-HT--$_{1A}$ receptor peptide corresponding to at least a portion of a functional domain of a 5-HT--$_{1A}$ receptor.

22. A method according to claim 21, wherein said 5-HT--$_{1A}$ receptor antibody is a an anti-peptide antibody against a functional domain of a 5-HT--$_{1A}$ receptor.

23. A method according to claim 3, wherein said antagonist is a monoclonal 5-HT--$_{1A}$ receptor antibody.

24. A method according to claim 23, wherein said 5-HT--$_{1A}$ receptor antibody is a an anti-peptide antibody against a functional domain of a 5-HT--$_{1A}$ receptor.

25. A method according to claim 14, wherein said antagonist is a monoclonal 5-HT--$_{1A}$ receptor antibody.

26. A method according to claim 25 wherein said 5-HT--$_{1A}$ receptor antibody is a an anti-peptide antibody against a functional domain of a 5-HT--$_{1A}$ receptor.

27. A method according to claim 20, wherein said antagonist is a monoclonal 5-HT--$_{1A}$ antibody.

28. A method according to claim 27, wherein said 5-HT--$_{1A}$ receptor antibody is a an anti-peptide antibody against a functional domain of a 5-HT--$_{1A}$ receptor.

29. A method according to claim 1, wherein prior to said contacting, the method further comprises the step of up-regulating the expression of 5-HT--$_{1A}$ receptors on central serotonergic neurons in the brain of said subject.

30. A method according to claim 3, wherein prior to said contacting, the method further comprises the step of up-regulating the expression of 5-HT--$_{1A}$ receptors on central serotonergic neurons in the brain of said subject.

31. A method according to claim 14, wherein prior to said contacting, the method further comprises the step of up-regulating the expression of 5-HT--$_{1A}$ receptors on central serotonergic neurons in the brain of said subject.

32. A method according to claim 20, wherein prior to said administering, the method further comprises the step of up-

regulating the expression of 5-HT--$_{1A}$ receptors on central serotonergic neurons in the brain of said subject.

33. A method for stimulating growth of central serontonergic neurons in a subject, comprising administering to said subject a serotonergic neuron stimulating effective amount of a 5-HT--$_{1A}$ receptor agonist.

34. A method according to claim 33, wherein said 5-HT--$_{1A}$ receptor agonist is selected from the group consisting of 5-hydroxytryptamine, 5-methoxytryptamine, buspirone, 8-hydroxydipropylamineotetralin, ipsaspirone, gepirone, SM23997, lysergic acid diethylamide and a 5-HT--$_{1A}$ receptor agonistic antibody.

FIG. 1

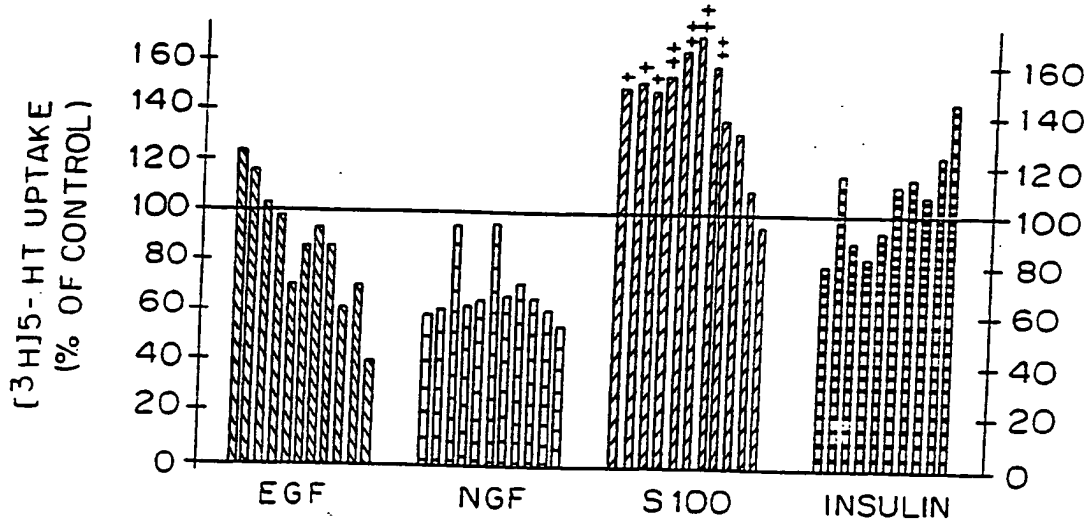

[3H]5-HT UPTAKE
(% OF CONTROL)
160
140
120
100
80
60
40
20
0
EGF
NGF
S100
INSULIN

FIG. 2

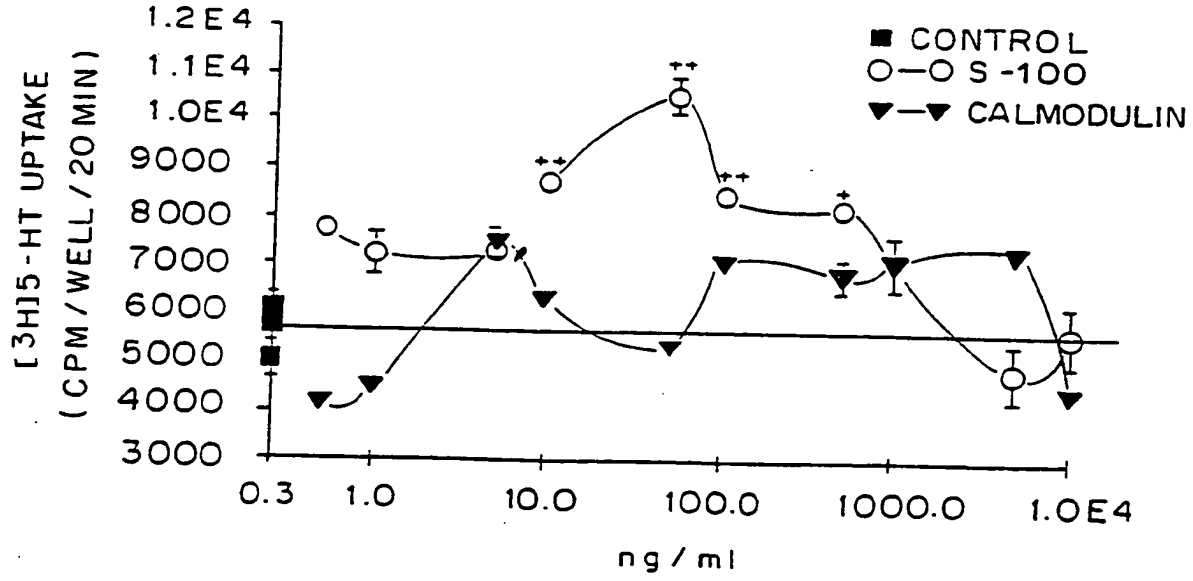

[3H]5-HT UPTAKE
(CPM / WELL / 20 MIN)
1.2E4
1.1E4
1.0E4
9000
8000
7000
6000
5000
4000
3000
CONTROL
S-100
CALMODULIN
0.3
1.0
10.0
100.0
1000.0
1.0E4
ng / ml

FIG. 3

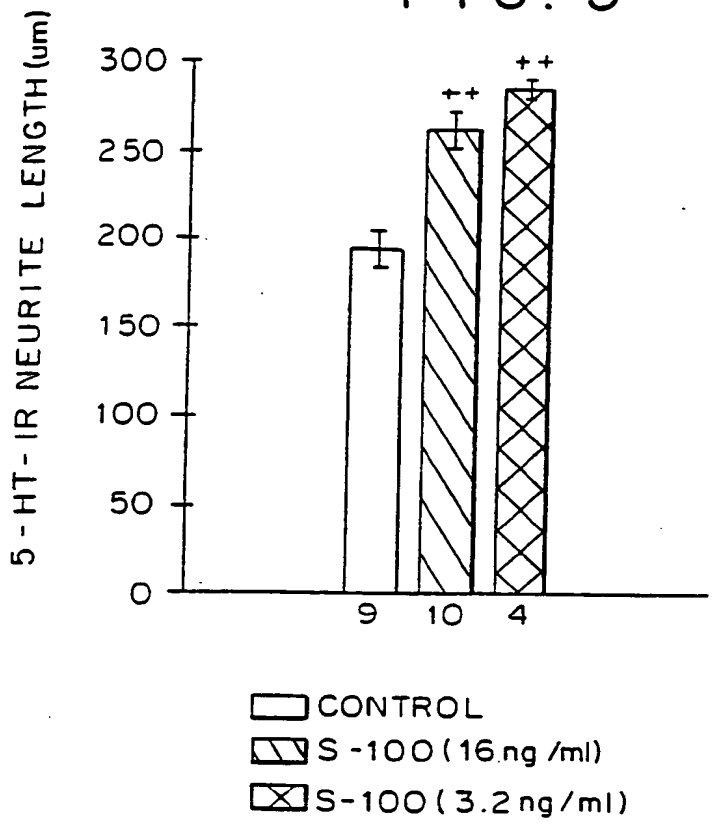

5-HT-IR NEURITE LENGTH (um)
300
250
200
150
100
50
0
++
++
9
10
4
CONTROL
S-100 (16 ng/ml)
S-100 (3.2 ng/ml)

FIG. 4

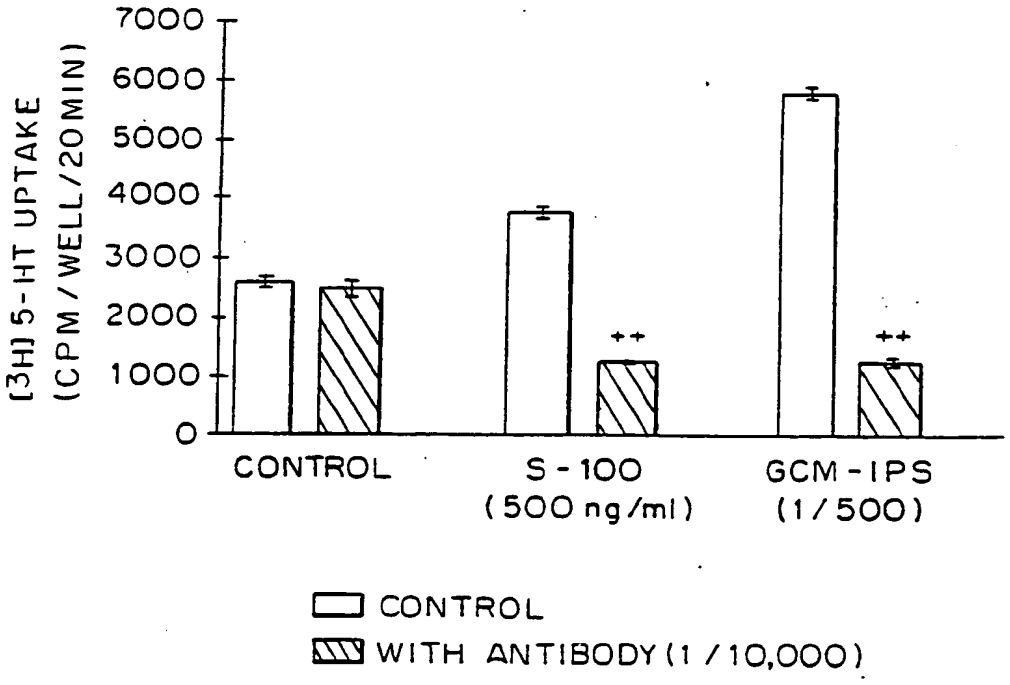
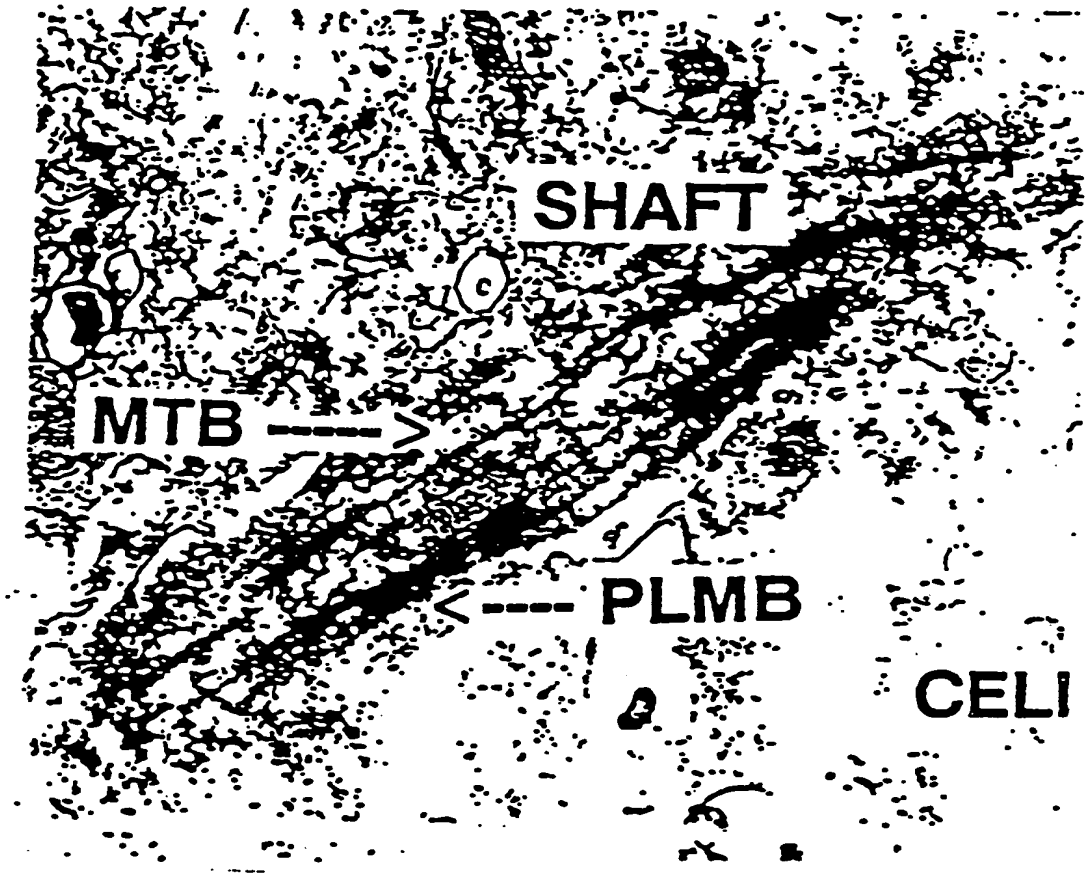

[3H] 5-HT UPTAKE (CPM/WELL/20MIN)
7000
6000
5000
4000
3000
2000
1000
0
++
++
CONTROL
S-100 (500 ng/ml)
GCM-IPS (1/500)
CONTROL
WITH ANTIBODY (1/10,000)

FIG. 5



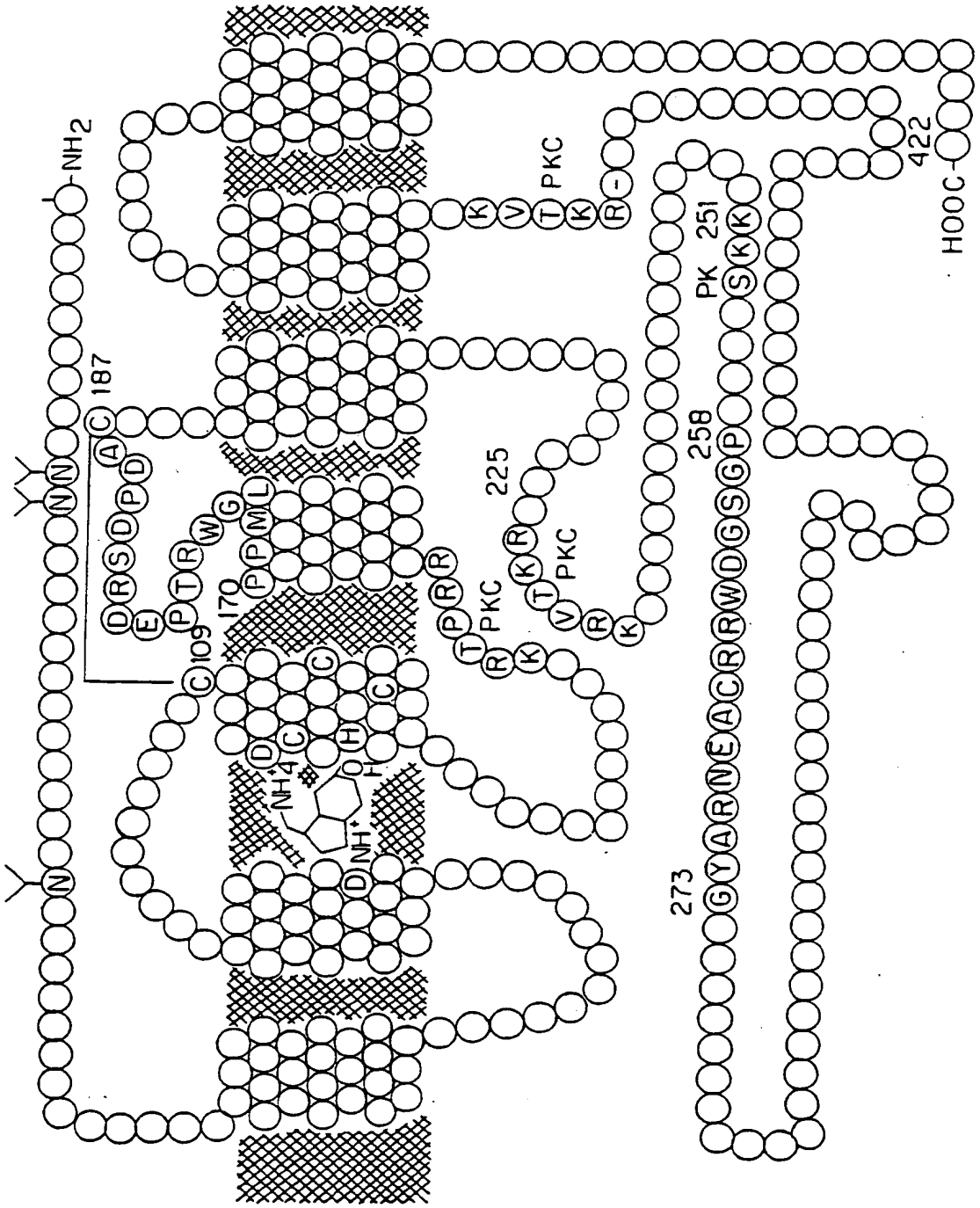


FIG. 6

FIG. 7

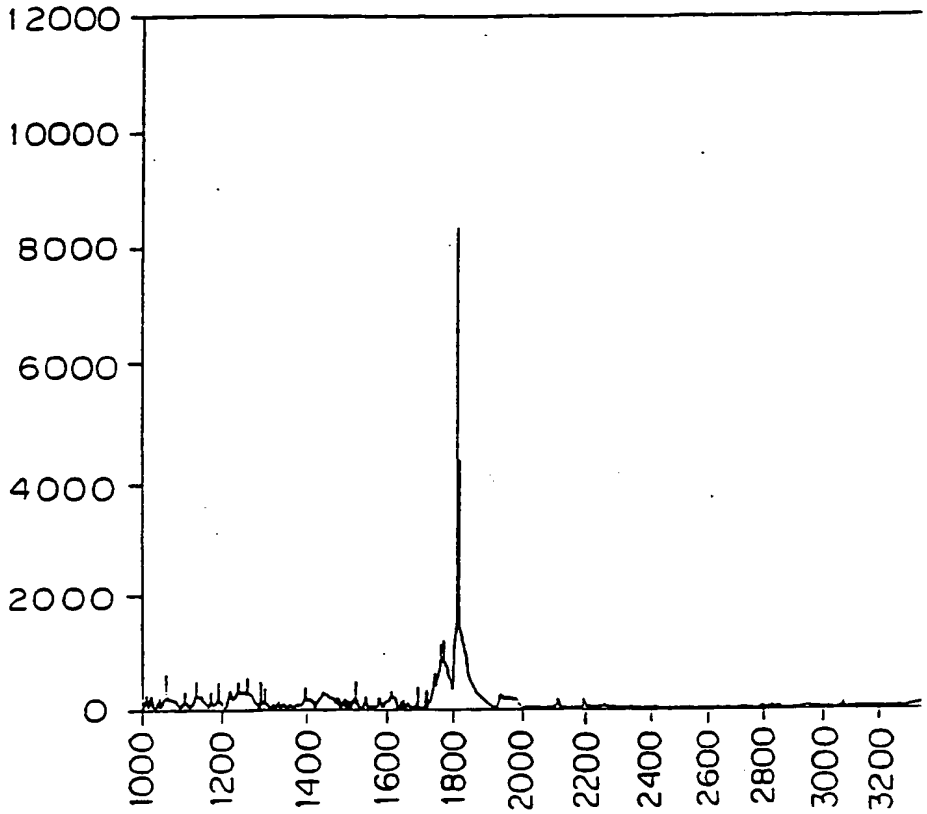
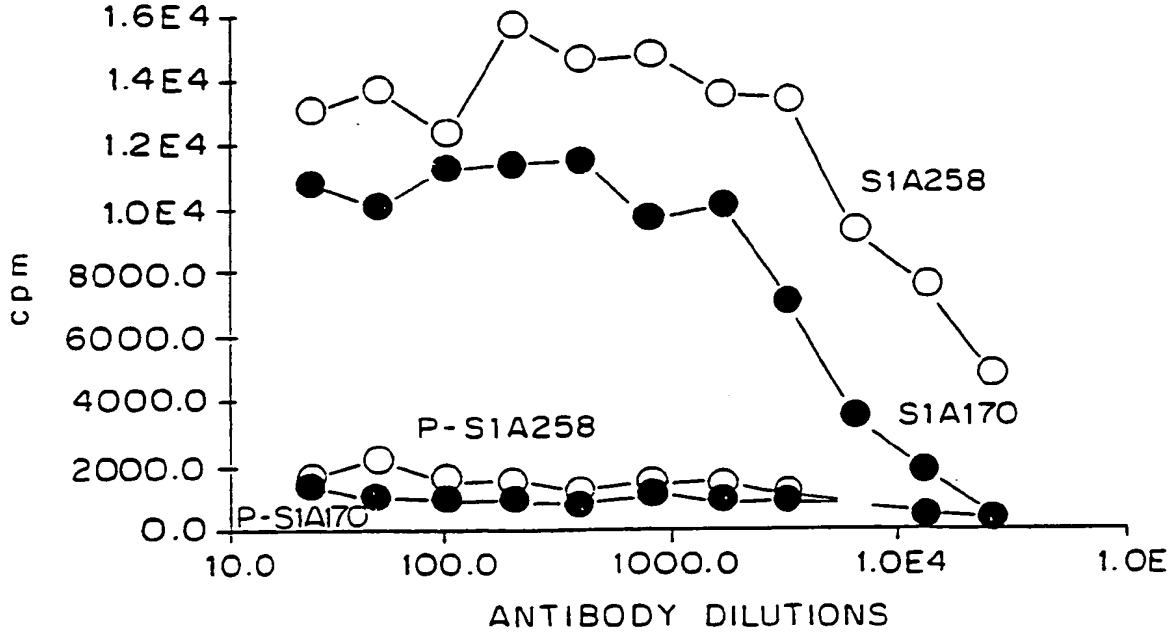


FIG. 8



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10095

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 31/00, 31/50, 31/60, 31/495
US CL :514/159, 252

According to International Patent Classification (IPC) or to both nati nal classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/159, 252

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and MEDLINE (files 5, 155, 351, 357, 358) search terms: ipsapirone, serotonin, 5-HT1A receptor, S100B

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	PSYCHOPATHOLOGY, Volume 22, Supplement 1, issued April 1989, J.P. Feighner et al., "Serotonin-1A Anxiolytics: An Overview," pages 21-26.	1-2, 14-16 29, 31
X Y	PSYCHOPATHOLOGY, Volume 22, Supplement 1, issued April 1989, M.S. Eison, "The New Generation of Serotonergic Anxiolytics: Possible Clinical Roles," pages 13-20.	1-2, 14-16 29, 31
X Y	US, A, 5,070,102 (TRABER ET AL) 03 DECEMBER 1991, see column 1, lines 12-35, and column 3, lines 15-40.	1-2, 14-16 29, 31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 JANUARY 1994	19 JAN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer MARIANNE PORTA ALLEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10095

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P Y,P	US, A, 5,254,552 (ABOU-GHARBIA ET AL) 19 OCTOBER 1993, see column 1, lines 21-40.	1-2, 14-16 29, 31

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10095

Box I Observations wher certain claims were found unsearchable (Continuation of item 1 f first sheet)

This international report has not been established in respect f certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Group I, specie A, which corresponds to claims 1-2, 14-16, 29, 31

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10095

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-2, 14-16, 21-22, 25-26, 29, and 31, drawn to a method for stimulating the production or release of S-100_B in a subject, and to a method for treating a disease associated with decreased central serotonergic innervation, classified in class/subclass 514/1+ and 424/85.8, for example.

II. Claims 3-7, 20, 23-24, 27-28, 30, and 32-34, drawn to a method for stimulating growth of central serotonergic neurons in a subject, classified in class/subclass 514/1+ and 424/85.8, for example.

III. Claims 8-13, drawn to a method of inhibiting the growth of central serotonergic neurons, classified in Class/subclass 435/240.1, for example.

IV. Claims 17-19, drawn to a method for treating a disease associated with increased central serotonergic innervation, classified in Class/subclass 514/1+ and 424/85.8, for example.

The groups also embrace the following species which are distinct from one another because they are different reagents with different structures and functional properties. Some are proteins and others are chemicals.

Group I, species A-K:

Species A - ipsapirone (claims 1-2, 14-16, 29, 31)
Species B - 5-methoxytryptamine
Species C - buspirone
Species D - 8-hydroxydipropylaminotetralin
Species E - 5-hydroxytryptamine
Species F - gepirone
Species G - SM23997
Species H - lysergic acid diethylamide
Species I - an agonistic antibody
Species J - S-100_B and functional derivatives
Species K - a 5-HT_{1A} receptor antibody

Group II, species A-K:

Species A - ipsapirone
Species B - 5-methoxytryptamine
Species C - buspirone
Species D - 8-hydroxydipropylaminotetralin
Species E - 5-hydroxytryptamine
Species F - gepirone
Species G - SM23997
Species H - lysergic acid diethylamide
Species I - an agonistic antibody
Species J - S-100_B and functional derivatives
Species K - a 5-HT_{1A} receptor antibody

Group III, species A-E:

Species A - an antibody specific for S-100_B
Species B - a 5-HT_{1A} receptor antagonistic antibody
Species C - a 5-HT_{1A} receptor antagonistic peptide
Species D - spiperone
Species E - spiroxatine

Group IV, species A-B:

Species A - an antibody specific for S-100_B
Species B - a 5-HT_{1A} receptor antagonist

The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to f rm

a single inventive concept. The special technical feature is the $S\text{-}100_B$ protein which would have been known in the art. The inventive contributions are the various methods of modulating the protein (see groups I-IV) and the agents (see the indicated species) used to do so.

The inventions of I-IV are materially different methods, involving different steps, reagents, and objectives. The species are distinct from one another because they are different reagents with different structures and functional properties.